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Mechanisms of immune activation and regulation in chronic inflammatory diseases of the gut.

BY

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Abstract

The gastrointestinal (GI) tract is the largest surface area of the body exposed to the external environment and it is subject to constant challenge from micro-organisms and food-derived antigens. Stringent regulation of the immune system of the gut is vital, as breakdown in tolerance to commensal bacteria in Inflammatory Bowel Diseases (IBD, such as Crohn's disease and Ulcerative Colitis (UC)) or to dietary antigens such as gluten in Celiac disease (CD) can lead to inflammation. Hence induction and maintenance of mucosal tolerance is of paramount importance to avoid inappropriate immune responses within the intestine.

The aim of this thesis was to investigate the mechanisms of activation and regulation in these intestinal autoimmune diseases. Firstly, I have investigated the mechanisms of immune regulation of mucosal tolerance looking at the role of regulatory T cells such as $CD4^+CD25^+FOXP3^+$ T cells in IBD and CD. Here I show that anti TNF-α treatment *in vivo* with Infliximab in Crohn's patients does not solely neutralise TNF-α, but also affects activation and possibly expansion of mucosal regulatory T cells. I suggest that anti TNF-α immunotherapy can also restore mucosal homeostasis in Crohn's disease. Furthermore, I show that anti IL-2 Receptor (CD25) treatment *in vivo* in UC down-regulates the frequency of mucosal regulatory T cells with an improvement of the disease. In CD I observed that antigenic challenge *in vitro* with gluten up-regulates regulatory T cells but despite this, mucosal homeostasis is not restored. My observations show how complex is the homeostatic balance of regulatory T cells response in different GI pathologies.

Next I have investigated the mechanisms of activation of the innate immune response that induce modifications like actin reorganization, protein tyrosine phosphorylation and apoptosis of the epithelial cells in CD. I have therefore explored which signalling pathway on epithelial cells might be involved in these early responses involving the innate immune system. The role of the small GTPase Rho-A pathway has been investigated as possible candidate in modulating these modifications. Here I demonstrate the role of Rho-A GTPase pathway in the innate immune response to gluten with epithelial cell modifications in CD.

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Abbreviations

2-ME	2-mercaptoethanol
ADP	Adenosine diphosphate
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CARD	Caspase recruitment domain protein
CD	Celiac disease
CsA	Cyclosporin A
CTLA-4	Cytotoxic T lymphocyte antigen-4
dH_2O	Distilled water
DC	Dendritic cells
DPEC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FAE	Follicle associated epithelium
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissues
GAP	GTPase-activating proteins
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GI	Gastrointestinal
GITR	Glucocorticoid-induced TNFR family-related gene (GITR)
GTP	Guanosine triphosphate
HBSS	Hank's buffered salt solution
HIFCS	Heat-inactivated fetal calf serum
HLA	Human leukocyte antigen
IBD	Inflammatory Bowel Disease
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin

IL-2 receptor	IL-2R
ITAM	Immunoreceptor tyrosine-based activation motifs
JAK	Janus tyrosine kinase
kD	Kilo Daltons
LP	Lamina propria
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAP	Mitogen-associated protein
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NK	Natural Killer
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated Molecular Pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PI	Propidium Iodide
PRR	Pattern Recognition Receptor
pTyr	phospho Tyrosine
RT-PCR	Reverse transcription polymerase chain reaction
SCID	Severe combined immunodeficiency
SEB	Staphylococcus Enterotoxin B
SED	Subepithelial dome
SH3	Src homology 3
STATs	Signal transducers and activators of transcription
TAP	Transporter associated with antigen presentation
TCR	T-cell receptor
Th	T-helper cell
TLR	Toll Like Receptor
TJ	Tight junctions
TNF-α	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor-beta
Tr1	T regulatory 1
Tregs	T regulatory cells
TRITC	Tetramethyl Phodamine Iso-Thiocyanate
tTG	Tissue-Transglutaminase
UC	Ulcerative Colitis

Chapter 1 – General Introduction

1 Introduction

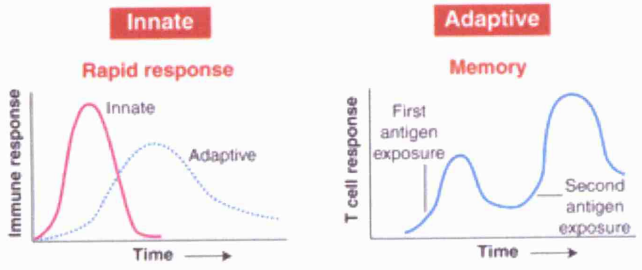
One primary function of the immune system is to protect the host from pathogens which threaten the host with a large spectrum of pathologic mechanisms. Central to the immune system's ability to mobilize a response to an invading pathogen is its ability to distinguish between "self" and "non self". The host's immune response therefore has evolved a complex array of protective mechanisms to control and usually eliminate such stimuli. All these mechanisms rely on the detection of structural features that are based on the recognition of molecular patterns demarcating pathologic or infectious non self, as well as normal and abnormal self (Medzhitov and Janeway, Jr., 2002). The immune system uses many different effector mechanisms to destroy the broad range of microbial cells and particles that it encounters. It is critical for this immune response to avoid unleashing these destructive mechanisms against its own tissues. This avoidance of destruction of self-tissues is referred to as self-tolerance. Failure of self-tolerance underlies the broad class of autoimmune diseases.

The immune system can be classified into two subsystems: the innate and the adaptive immune systems. Innate and adaptive immunity can be distinguished on the basis of their levels of specificity, with the antigen receptors of the adaptive immune system mediating highly specific responses, in contrast to the more promiscuous, and therefore less specific, recognition of pathogens by the innate immune receptors (Vivier and Malissen, 2005).

The innate immune system encompasses a collection of host defences that range from the non-specific barrier function of epithelia to the highly selective recognition of pathogens through the use of germ-line encoded receptors. Because the recognition molecules, used

by the innate immune system are expressed broadly on a large number of cells, this system is poised to act rapidly after an invading pathogen is encountered and thus constitutes the initial host response (Janeway, Jr. and Medzhitov, 2002). In the intestine, maintenance of this immediate-alert system is extremely important, but slight derangements may cause inappropriate immune reactions to normally harmless dietary antigens and bacteria of the commensal flora.

In contrast, the adaptive immune system is composed of small numbers of cells (lymphocytes) with specificity for individual pathogens; the responding cells must proliferate after encountering the pathogen to attain sufficient numbers to mount an effective response against the antigen (Janeway, Jr. and Bottomly, 1994). It takes three to five days for a sufficient number of lymphocytes to be produced and to mount an appropriate immune response, which is more than enough time for most pathogens to invade and damage the host. In contrast, innate immunity responds immediately to a wide variety of micro-organisms and can be seen as a first line of defence to control an invasion before clonal lymphocytes can mount a specific attack. Thus, the adaptive response generally expresses itself temporally after the innate response in host defence (**Figure 1.1**).



R. L. Modlin, P. A. Sieling, *Science*, 2005.

Figure 1.1 - The dynamics of innate and adaptive immune responses
The innate immune system acts rapidly after an invading pathogen is encountered and thus constitutes the initial host response. The adaptive immune response is slower but more flexible and is able to combat infections that have evolved to evade innate responses.

1.1 Innate immune system

The innate immune system recognises invariant and conserved molecular constituents of the infectious agent shared by a large group of pathogens.

This innate immune recognition is mediated by germ-line–encoded receptors, which means that the specificity of each receptor is genetically predetermined. One advantage of these germ-line–encoded receptors is that they have evolved by natural selection so as to have defined specificities for infectious microorganisms (Medzhitov and Janeway, Jr., 2000).

The cellular components of the innate immune system include barrier mechanisms, such as epithelial cell layers that express tight cell-cell contacts (tight junctions), cadherin-mediated cell interactions, and others), the secreted mucus layer that overlays the epithelium in the respiratory, gastrointestinal, and genitourinary tracts as well as dendritic cells, monocytes, macrophages, granulocytes and natural killer T cells. The innate immune system is also able to recognize soluble proteins and bioactive small molecules that are either constitutively present in biologic fluids (e.g., the complement proteins and defensins), or are released from cells as they are activated (including cytokines that regulate the function of other cells, chemokines that attract inflammatory leucocytes). This innate response is particularly important for mucosal surfaces that are continuously exposed to external pathogens or food antigens, like the intestinal mucosa (MacDonald and Pettersson, 2000).

1.1.1 Pathogen-associated molecular pattern (PAMP)

The innate immune system has evolved to recognize molecular patterns common to many classes of pathogen; these elements have been termed pathogen-associated molecular patterns (PAMP) (Janeway, Jr. and Medzhitov, 2002). The best known examples of PAMPs include bacterial lipopolysaccharide (LPS), peptidoglycan, mannans, bacterial

DNA, double-stranded RNA, and glucans. These and other PAMPs are recognized by receptors of the innate immune system called pathogen-recognition receptors (PRR) (Janeway, Jr. and Medzhitov, 2002). PRR were initially defined as cell surface pathogens receptors present on innate immune cells, but this definition has been expanded to include secreted and locally produced molecules that mediate many steps in inflammation including directed phagocytosis, activation of inflammatory signalling pathways, induction of cell death and activation of the complement or coagulation cascades (Janeway, Jr. and Medzhitov, 2002). The expression of pattern-recognition receptors is not clonal, in that all such receptors displayed by cells of a given type (e.g., macrophages) have identical specificities. Moreover, once the pattern-recognition receptors identify a pathogen-associated molecular pattern, the effector cells are triggered to perform their effector functions immediately without the need for a cell proliferative response. This accounts for the rapid kinetics of the innate immune response. Although PAMPs are chemically quite distinct, all these structures have common features. First, PAMPs are produced only by microbial pathogens, and not by their hosts. For example, LPS is synthesized only by bacteria; PRRs recognize LPS, thus alerting the host to the presence of the infecting organism. Second, the structures recognized by the innate immune system are usually essential for the survival or pathogenicity of microorganisms. Third, PAMPs are usually invariant structures shared by entire classes of pathogens. For example, all gram-negative bacteria have LPS, and therefore, the LPS-PRR of the host can detect the presence of virtually any gram-negative bacterial infection. Signalling receptors recognize PAMPs and activate signal-transduction pathways that induce the expression of a variety of immune-response genes, including inflammatory cytokines.

1.1.2 Toll like Receptors

The Toll-like receptors (TLRs) are a particularly important group of pathogen receptors which appear to have a major role in the induction of immune and inflammatory responses (Kaisho and Akira, 2000). These molecules are expressed by both innate immune cells and by cells of other tissues including endothelial cells, epithelial cells and fibroblasts (Janeway, Jr. and Medzhitov, 2002). TLRs are named after the Drosophila Toll gene, whose protein product, Toll, participates in innate immunity and in dorso-ventral development in the fruit fly. Analysis of the sequence of the *toll* gene revealed it to encode a transmembrane protein with a large extracellular domain containing leucine-rich repeats. Remarkably, the sequence of the cytoplasmic domain of the toll protein turned out to be similar to the cytoplasmic domain of the mammalian interleukin-1 receptor. Moreover, both the interleukin-1 receptor in humans and TLR in drosophila and in humans, when bound by an appropriate ligand, trigger activation of signal-transduction pathways that lead to the activation of transcription factors of the nuclear factor-κB (NF-κB) family. This signalling pathway is a master switch for the induction of inflammation.

Despite the limited spectrum of ligands recognized by each of the 13 known mammalian TLRs, the TLR repertoire probably detects most, if not all, microbes (Beutler, 2004; Ulevitch, 2004). The breadth of TLR recognition may well be due to the recognition of conserved motifs shared by most microorganisms. Binding of TLR by their microbial ligands leads to activation of phagocytes, direct killing of pathogens, and the release of pro-inflammatory cytokines and anti-microbial peptides. In addition, TLR activate dendritic cells and are therefore important in the initiation of adaptive immune responses.

1.1.3 Nucleotide-binding oligomerization domain (NOD)

Much as Drosophila was essential for unravelling the extra cellular system of TLRs, plants represent the model system for the discovery of intracellular (cytosolic) sensing system for microbial effectors that later led to the discovery of NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs) in mammals (Inohara and Nunez, 2003). Mammalian Nod proteins sense the presence of intracellular muropeptides derived from phagocytosed bacteria (Girardin et al., 2003) .

NOD1 and NOD2 recognize with an exquisite specificity ligands derived from bacterial peptidoglycans, with only small differences distinguishing NOD1 ligands (muramyl tripeptide structures in which the terminal amino acid is diaminopimelic acid) from NOD2 ligands (muramyl dipeptide structures).

This "global network" of bacterial sensors also seems to be essential for the maintenance of epithelial barriers in the presence of commensal microorganisms as opposed to pathogens. This is particularly evident in the intestinal mucosa, where the molecular network functions not only to protect against pathogen intrusion but also to strengthen the epithelial barrier and support development of the mucosa associated immune system (Sansonetti, 2006). It is likely that the need to maintain and tolerate the beneficial gut flora, which can achieve enormous numbers in the colon ($1X10^{11}$ to $1X10^{12}$ bacteria per gram of stool) and which present a permanent "threat of invasion" after mechanical or immunological breach, has forged a very robust innate immune network (Backhed et al., 2005). Indeed, the very strength of the innate network poses a fascinating immunological paradox: sensing and responding to pathogens must be pro-inflammatory, and thus destructive, whereas sensing

and responding to commensal bacteria must be tolerogenic, unless the "barrier" is breached (Sansonetti, 2004).

Additionally NOD proteins are also critical for normal intestinal biology and pathogen responses. NOD1 deficient mice, for example, are unable to properly regulate their gastric colonization by *Helicobacter pylori* (Viala et al., 2004). However, the best evidence for the involvement of Nod molecules in the global sensing of microbes and maintenance of gut homeostasis is the observation that mutations in the gene encoding NOD2 that reduce recognition of the PAMP muramyl dipeptide are associated with cases of familial Crohn's disease in some populations (Hugot et al., 2001; Ogura et al., 2001). In support of the idea of an integrated innate immune network in gut biology, accumulating evidence indicates that the TLR and NOD systems engage in signalling crosstalk, which probably accounts for the reciprocal regulation and thus explains how a lack of NOD function causes a pro-inflammatory situation as in Crohn's disease (Kufer and Sansonetti, 2007). Related to that issue is the need to understand why pathogens, after being sensed by he host, elicit inflammation, whereas commensal bacteria essentially elicit tolerance. It does not seem to be the case that bacterial PAMP motifs are so different in pathogens versus commensals that they would be sensed or ignored, respectively. The fact brings to the fore the possibility of referring to pathogen motifs as "MAMPs" ("microbe-associated molecular patterns"), instead of PAMPs: both commensals and pathogens share such motifs, so why are they sensed differently by the host? It is likely that the borderline between commensals and pathogens resides mainly in the capacity of the latter to gain close access to the surface epithelium and associated immune cells and to deliver enzymes and toxins that disrupt barriers and facilitate access of PAMPs to their receptors. Also, through their capacity to

alter cells, pathogens are able to induce the release of "danger signals"(Gallucci and Matzinger, 2001).

1.2 Adaptive immune system

Unlike innate mechanisms of host defence, the adaptive immune system manifests exquisite specificity for its target antigens. Adaptive responses are based primarily on the antigen-specific receptors expressed on the surfaces of T and B lymphocytes. T and B cells undergo a recombination of antigen receptor genes to create novel and unique antigen receptors capable of recognizing virtually any antigen in a specific manner.

In the adaptive immune system, the T-cell receptor and the B-cell receptor are generated somatically, during the development of T and B cells, in a way that endows each lymphocyte with a structurally unique receptor. Since these receptors are not encoded in the germ line, they are not predestined to recognize any particular antigen. Rather, an extremely diverse repertoire of receptors is generated randomly, and lymphocytes bearing useful receptors (i.e., receptors specific for pathogens) are subsequently selected for clonal expansion by encountering the antigen for which they happen to be specific. These useful receptors, moreover, cannot be passed on to the next generation, even though they might give one's progeny a survival advantage. No matter how beneficial they may be, antigen receptors for common environmental pathogens have to be reinvented by every generation (Medzhitov and Janeway, Jr., 2000).

A key feature of the adaptive response is that it produces long-lived cells that persist in an apparently dormant state, but that can re-express effector functions rapidly after repeated encounter with antigen. This provides the adaptive response with the ability to manifest immunologic memory, permitting it to contribute to a more effective host response against

specific antigens when they are encountered a second time, even decades after the initial sensitizing encounter.

Adaptive immune responses require B cells, to provide antibody, and T cells, to provide cell-mediated immunity.

1.2.1 B cells

B cells are formed in the bone marrow and produce antibodies. Antibodies, encoded by heavy and light immunoglobulin (Ig) genes, are the antigen receptors on B cells. Antibodies are generated from the cutting and splicing of immunoglobulin genes early in B-cell development, and have the potential to develop strong and highly specific affinity for different pathogen's epitopes. The stages of B-cell development are marked by a series of irreversible changes in the immunoglobulin genes, which contribute to the diversity of antibodies. Selected B cells then differentiate into plasma cells, which secrete large amounts of antibody, or long-lived memory cells, which contribute to lasting protective immunity. Many IgG-secreting plasma cells migrate to the bone marrow, while B cells that have differentiated to secrete IgA migrate to the lamina propria of the mucosal surfaces (Clark and Kupper, 2005).

IgA is the main element of the humoral immune response that has been selected through evolution, together with innate mucosal defences, to provide protection against microbial antigens at mucosal surfaces. The gut, where the synthesis of IgA is very abundant, is responsible for the most extensive immunoglobulin production of the body, containing up to 70% to 80% of the body's total Ig-producing tissue (Brandtzaeg, 1989).

1.2.2 T cells

T cells are defined by their cell surface expression of the T cell receptor (TCR), a transmembrane heterodimeric protein that binds processed antigen, displayed by antigen presenting cells (APC).

Selection of cells carrying functional TCR genes occurs in the thymus, a complex lymphoid organ located in the anterior mediastinum at the base of the neck (Miller, 2002).

Each TCR consist of two different polypeptide chain, termed the T-cell receptor alpha (TCRα) and beta (TCRβ) linked by a disulfide bond. A minority of T cells bear alternative, but structurally similar, receptor made of a different pair of polypeptide chains designated γ and δ. The γ/δ TCRs seem to have different antigen recognition properties from the α/β TCR and the function of γ/δ T cells in immune responses is not yet entirely clear. Both types of TCR differ from the membrane bound Ig that serve as the B-cell receptor. A T cell receptor has only one antigen–binding site, whereas a B-cell receptor has two sites.

Approximately 90% to 95% of circulating T cells uses the αβ TCR. The other 5% to 10% use γδ TCR. A portion of the γδ T cells is generated in the thymus, but a major fraction appears to be generated in an extra-thymic compartment. The gastrointestinal (GI) tract contains predominantly T cells with a γδ TCR (McVay and Carding, 1999).

The antigenic-specific α and β chains of the TCR associate with invariant accessory chains that serve to transduce signals when the TCR binds to antigen-MHC complexes (Peterson et al., 1998). These accessory chains make up the CD3 complex, consisting of the transmembrane CD3γ, CD3δ, CD3ε chains, plus a largely intra-cytoplasmic homodimer of two CD3ζ chains. The stoichiometry of the CD3 complex is not definitely established, but it appears that each TCR αβ pair is associated with a CD3γε heterodimer, a CD3δε

heterodimer, and a CD3ζ homodimer. The cytoplasmic portions of each of the CD3 chains contain sequence motifs designated ITAMs (immunoreceptor tyrosine-based activation motifs) **(Figure 1.2)**. When key tyrosines in these ITAMs are phosphorylated by the receptor-associated kinases Lck and Fyn, this initiates an activation cascade involving the proteins ZAP-70, LAT, and SLP-76. Activation of these proteins leads to stimulation of phospholipase C, activation of G proteins Ras and Rac, and both protein kinase C and the mitogen-associated protein (MAP) kinases. Together, this complex of activation events leads to activation of genes that control lymphocyte proliferation and differentiation.

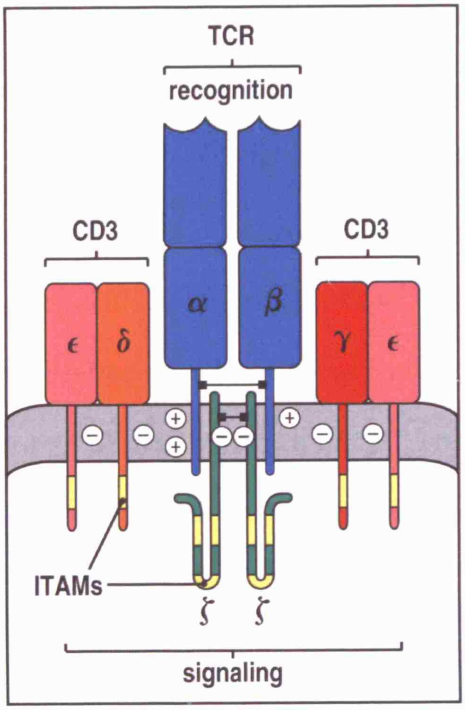


Figure 6-9 Immunobiology, 6/e. (© Garland Science 2005)

Janeway, Travers et al. *Immunobiology* 6th Edition, 2005

Figure 1.2 The T Cell Receptor (TCR) complex
The antigenic-specific α and β chains of the TCR associate with invariant accessory chains (CD3) that serve to transduce signals when the TCR binds to antigen-MHC complexes. This signal induces T cell activation and proliferation.

During their progress through the thymus, αβ T cells differentiate into discrete subpopulations, each with defined repertoires of effector functions. The major subsets are defined by their selective surface expression of CD4 or CD8. In the thymus, most developing T cells follow a developmental program in which, in the cortex, they first express neither CD4 nor CD8 (double negative, DN), and then express both CD4 and CD8 (double positive, DP) (von et al., 1989). DP cells are tested by positive selection in the thymic cortex, those that are selected on class I MHC molecules become $CD4^{-}CD8^{+}$, and those that are selected on class II MHC molecules become $CD4^{+}CD8^{-}$, then move to the thymic medulla for negative selection and export to the periphery. In the blood and secondary organs lymphoid organs, 60% to 70% of T cells are $CD4^{+}CD8^{-}$ ($CD4^{+}$) and 30% to 40% are $CD4^{-}CD8^{+}$ ($CD8^{+}$). $CD4^{+}$ cells are generally designated "helper cells" and work to activate both humoral immune responses (B-cell help) and cellular responses (delayed-type hypersensitivity responses). $CD8^{+}$ cells show a major cytotoxic activity against cells infected with intracellular microbes and against tumour cells.

A major role of the T cell arm of the immune response is to identify and destroy infected cells. T cells with their TCR can recognize peptide fragments of antigens that have been taken up by Antigen Presenting Cells (APC) by phagocytosis or pinocytosis. APCs present antigens (Ags) to T cells, through Major Histocompatibility Complex (MHC) molecules. MHC molecules, also called the human leukocyte-associated antigens (HLA), are cell-surface glycoproteins that bind peptide fragments of proteins that either have been synthesized within the cell (class I MHC molecules) or that have been ingested by the cell and proteolytically processed (class II MHC molecules).

The MHC has evolved in terms of variability between individuals with a highly polymorphic system based on multiple alleles (i.e. alternative genes at each locus). The class I and class II genes are the most polymorphic genes in the human genome; for some of the genes over 200 allelic variants have been identified.

1.2.3 Class I MHC molecules

There are three major HLA class I molecules, designated HLA-A, -B, -C, each encoded by a distinct gene. The class I HLA molecules are cell-surface heterodimers, consisting of a transmembrane polymorphic heavy polypeptide α-chain of 44-kDa not covalently linked to a smaller 12-kDa nonpolymorphic β_2-microglobulin protein (Bjorkman, 1997). The α-chain determines whether the class I molecule is an HLA-A, -B, -C molecule. The HLA-A, -B, -C α-chain genes are encoded within the MHC on chromosome 6 and the β_2-microglobulin gene is encoded on chromosome 15.

The fact that there are three distinct HLA class I genes and that each is highly polymorphic means that all individuals in the population who are heterozygous at these loci have six distinct peptide-binding grooves. Generally, the antigenic peptides that are found bound in the peptide-binding groove of the HLA class I are derived from proteins synthesized within the cell that bears the class I molecules. They are, consequently, described as "endogenous" antigens. The peptides like for example, viral peptides produced in a virus-infected cell are delivered to cell surface bound to class I HLA molecules in a form that can be recognized by cytotoxic $CD8^+$ T cells.

1.2.4 Class II MHC molecules

Three major class II proteins are designated HLA-DP, HLA-DQ, and HLA-DR (Bjorkman, 1997). The MHC class II genes are also encoded on chromosome 6. These molecules consist of two non-covalently associated polypeptide chains: the α-chain and the β-chain, with a molecular weight of 34 kDa and 29 kDa, respectively. In addition to their extracellular regions, both chains have a single transmembrane sequence and a short cytoplasmic tail. The amino (N)-terminal α1 and β1 regions of the chains combine to form a membrane-distal peptide-binding domain, whereas the remaining extracellular portions of the two chains each form a membrane-proximal immunoglobulin-like domain (α2 and β2 regions). MHC class II molecules are expressed on the surface of epithelial cells in the thymus and on professional antigen-presenting cells in the periphery and present peptides to $CD4^+$ T cells (Konig et al., 1992). This provides the mechanism by which MHC class II molecules function in the maintenance of self-tolerance and in the induction and regulation of adaptive immune responses against invading pathogens (Jones et al., 2006).

Antigens that are presented by class II proteins are loaded into the class II peptide-binding grove by the "exogenous" pathway that starts by endocytosis or phagocytosis of extra-cellular proteins. The antigens include antigenic proteins of extra-cellular pathogens, such as most bacteria, parasites, and virus particles as well as food antigens. Side aminoacid chains at certain positions are important residues in the binding between the antigenic peptide and the MHC II. The residues at these positions in the peptide are termed anchor residues because the interactions of their side chains with distinctive pockets in the binding groove further stabilize the peptide–MHC-class-II complex.

1.2.5 Association of HLA types and disease susceptibility

A large number of chronic inflammatory diseases are associated with genes in the MHC class II region.

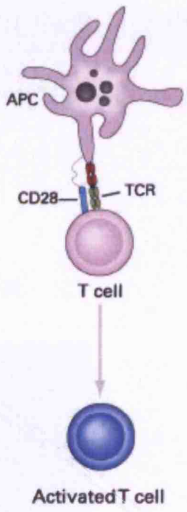
In Celiac Disease (CD) for example, an autoimmune-like disorder of the small intestine, caused by an immune response to antigens (such as α-gliadin) in wheat gluten, the vast majority of patients are either HLA-DQ2 positive (90%) or HLA-DQ8 (5%) (Sollid et al., 1989). Immunogenic gluten epitopes that are recognized by HLA-DQ2- or HLA-DQ8-restricted T cells have been identified, and these have sequences in common that are rich in proline and glutamine residues (Lundin et al., 1994; Lundin et al., 1993). It has recently been shown that deamidation of glutamine residues in these gluten peptides by the enzyme transglutaminase 2 (the expression of which is upregulated in inflamed intestines) generates epitopes that bind efficiently to HLA-DQ2 and HLA-DQ8 and are recognized by T cells isolated from lesions in the gut of patients with coeliac disease (Molberg et al., 1998). The link between gluten epitopes and coeliac disease is exemplified by the α-gliadin peptides (with an amino acid sequence PFPQPQLPY, called α9 and PQLPYPQPQLPY, called α2), which when selectively deamidated by transglutaminase 2 and presented by HLA-DQ2 as the aminoacid sequence PFPQPELPY, and PELPYPQPQLPY, respectively, induce potent T-cell responses and for this they are called immunodominant epitopes. These peptide have a central role in celiac disease pathogenesis because able to activate T cell responses (Molberg et al., 1998) that lead to the adaptive immune response against gluten.

1.2.6 Activation of T cells

The recognition of the peptide–MHC ligand by the antigen receptor is not sufficient to activate T cells. T cells require at least two signals to become activated: one is the complex

of a peptide and an MHC molecule, and the other is a co-stimulatory molecule, such as CD28, on the T cell, and CD80 or CD86 on the surface of the APC.

It is only when the APC expresses both antigen and CD80 or CD86 molecules that the T cell can be activated (Lenschow et al., 1996). In fact, interaction of peptide-MHC with the TCR without a co-stimulator factor can lead to an anergic state of prolonged T cell unresponsiveness (Guerder and Flavell, 1995).



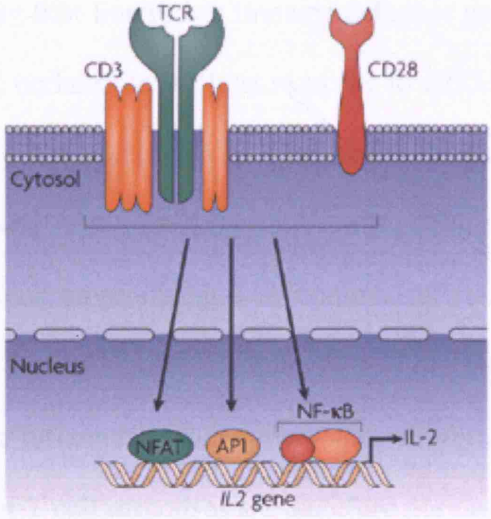
St Clair EW, *Annual Review of Medicine*, 2007.

Figure 1.3 T cell activation.

Normal T cell activation requires the interaction of MHC/peptide on APCs with the T cell receptor and the interaction of co-stimulatory molecules, most prominently CD80/86 on APCs with CD28 on T cell. The mechanisms of tolerance controls effective T cell activation through interfering with this interaction.

1.2.6.1 Th1 and Th2 effector T cells.

Both $CD4^+$ and $CD8^+$ T cells, after exposure to antigen and being activated, differentiate into functionally distinct subsets. This is best described for the transition of $CD4^+$ T cells from naïve to effector populations (Abbas et al., 1996). $CD4^+$ effector T cells undergo a stereotypical activation program after engagement of their TCR and appropriate co-stimulation. This program consists of the activation of specific signaling pathways that result in the induction of effector functions, including the production of IL-2 (Crabtree and Clipstone, 1994). The consequences of these signaling cascades include alterations in the transcriptional program and proliferation (Laouar and Crispe, 2000).



Campbell DJ, *Nature Review Immunol*, 2007.

Figure 1.4 – Signaling in effector $CD4^+$ T cells.
In normal T cell activation engagement of the T-cell receptor (TCR) and CD28 leads to activation of signaling pathways that culminate in the nuclear translocation of nuclear factor of activated T cells (NFAT), nuclear factor-κB (NF-κB), and activator protein 1 (AP1), and subsequent transcription of the IL-2 gene.

Resting naïve $CD4^+$ T cells (designated T helper cells, Th) release little detectable cytokine. Early after stimulation by antigen and APC, the Th cells begin to produce IL-2 and are designated Th0. As the Th cells continue to respond to the activated signal, they progress toward polar extremes of differentiation designated Th1 and Th2, depending on the nature of the cytokines present at the site of activation (Mosmann and Coffman, 1989). Th1 cells are characterized by the production of IL-2, IFN-γ, and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13, and granulocyte-macrophage colony stimulating factor. Generally, Th1 cells support cell-mediated immune responses, and Th2 cells support humoral and allergic responses. The divergence of Th1 and Th2 differentiation is largely due to cross-regulatory effects of these polarizing cytokines, providing a mechanism whereby first-line innate immune defences guide appropriate effector T cell responses that, in turn, orchestrate the host response to efficiently clear pathogens and establish long-lived memory for enhanced recall responses. Inappropriate or poorly controlled effector T cells can cause host pathology and are particularly deleterious when directed against self or ubiquitous environmental or commensal floral antigens, which, unlike most pathogens, cannot be effectively cleared. In this setting, persistent effector T cell responses drive chronic inflammatory disorders such as autoimmunity and inflammatory bowel diseases. Effector T cell responses are therefore normally under stringent regulatory control (Weaver et al., 2006). There has been extensive investigation of the factors and signalling pathways that distinguish differentiation of Th1 and Th2 cells (Murphy and Reiner, 2002). The process by which an uncommitted T cell develops into a mature Th1 or Th2 cell is a useful model of developmentally regulated gene expression. There is good evidence to indicate that this differentiation process is highly plastic. Many data indicate that certain crucial

transcription factors have causal roles in the gene-expression program of Th1 and Th2 cells. For example, presence of IL-12 promotes skewing towards Th1 commitment by signalling through signal transduction and activator of transcription STAT-4. Th1 cells are characterized by expression of the T-box transcription factor T-bet (Szabo et al., 2002) and produce interferon-γ (IFN-γ) and tumour necrosis factor–α (TNF-α). By contrast, Th2 cell commitment is promoted by IL-4 via STAT-6 signalling. Th2 cell committed cells are characterized by expression of the zinc-finger transcription factor GATA3 and produce IL-4, IL-5 and IL-13 (Zheng and Flavell, 1997).

1.2.6.2 Th17

Another class of effector T cells is represented by the Th17 lineage. Th17 cells are characterized by the production of a distinct profile of effector cytokines, including IL-17 (or IL-17A), IL-17F, and IL-6, and have probably evolved to enhance host clearance of a range of pathogens distinct from those targeted by Th1 and Th2. Th17 cells develop via a pathway separate from Th1 and Th2, but with several notable parallels to the Th1 lineage that have led to some confusion over the role of the Th1 cells in autoimmunity. The development of Th17 effectors also shares with some T regulatory cells (Tregs) (see below) a requirement for TGF-β, establishing an important link between Th17 and Treg development (Weaver et al., 2006). Th17 cells are characterized by expression of the RORγT transcription factor (Ivanov et al., 2006) and produce IL-17. The predominant function of IL-17 is to be a pro-inflammatory mediator through a variety of mechanisms. IL-17 induces expression of many innate inflammatory mediators, including IL-6, acute phase proteins, granulocyte-colony stimulating factor (G-CSF) and prostaglandin E2 (Komiyama et al., 2006). IL-17 also synergizes potently with inflammatory cytokines such

as TNF-α, amplifying its effects by orders of magnitude (Komiyama et al., 2006; McAllister et al., 2005). Thus, IL-17 is a means by which the adaptive immune system communicates with the innate immune system to promote inflammation.

1.3 Regulatory T cells (Tregs)

Besides effector subsets, CD4 T cells can differentiate into distinct regulatory subsets characterized by their ability to suppress effector pathogenic T cell responses and prevent autoimmunity (Sakaguchi, 2000). While at least one class of regulatory T cells, nTregs, develops intra-thymically, mounting evidence indicates that other Tregs develop from naive CD4 T cell precursors in the periphery, so-called induced, or adaptive, Tregs (aTregs).

To maintain the immunological tolerance, including intestinal homeostasis, functionally distinct subsets have been clearly defined in $CD4^{+}T$ cells (Abbas et al., 1996; O'Garra, 1998). Among these subsets, the regulatory T cell subset down-regulates immune responses for both foreign and self-antigens and effectively participates in the suppression of autoimmune disorders (Sakaguchi et al., 2001).

A variety of T reg cells that display regulatory function *in vitro* or *in vivo* have been described. These can be subdivided into different subsets based on the expression of cell surface markers, production of cytokines, and mechanisms of action.

One type of regulatory cell is a TGF-β secreting T cell (a so called Th3 cell), which is the cell induced by antigen feeding during the development of oral tolerance. The mucosal cytokine milieu necessary for the induction of this cell is not well understood, although it is known that Th2 conditions favour induction and Th1 conditions inhibit induction (Strober et al., 1998) .

IL-10 has been seriously considered as a possible inductive cytokine for this cell, but in recent studies of oral tolerance induction as well as *in vitro* studies of the development of Th3 cells from naïve cells, IL-10 has no direct inductive effect on the development of Th3 cells and may enhance TGF-β production only through its capacity to down-regulate Th1 responses.

A second type of regulatory T cell is an IL-10 secreting cell (Tr1 cell), which may also secrete small amount of TGF-β (Groux et al., 1997). This cell has poor proliferative capacities and in initial studies was induced sequential antigenic re-stimulation in the presence of IL-10. More recently, however, it has been shown that both IL-10 and IFN-γ are necessary for its induction (Levings et al., 2001).

A third type of regulatory cell is represented by the naturally occurring $CD4^+$ T regulatory cells that constitutively express the α chain of the interleukin-2R (CD25) (Sakaguchi, 2000) ($CD4^+CD25^+$ Tregs). $CD4^+CD25^+$ Treg cells comprise a minor population of $CD4^+$ T cells: on average 5~10% in rodents and humans (Coombes et al., 2005; Sakaguchi, 2005). $CD4^+CD25^+$ Tregs are the main focus of current research, because accumulating evidence indicates that this population plays a crucial role in the maintenance of immunological self tolerance and negative control of pathological as well as physiological immune responses. $CD4^+CD25^+$ Tregs are essential for the maintenance of self tolerance and their activation after oral antigen administration suggests that these cells may also be involved in oral tolerance (Zhang et al., 2001). $CD4^+CD25^+$ T cells may also have an important role in the tolerance towards the microbial flora, as suggested by their ability to prevent and cure colitis in mice with severe combined immunodeficiency (SCID) (Mottet et al., 2003).

1.3.1 Origin of $CD4^+CD25^+$ T regulatory cells

The normal thymus produces the majority, if not all, of $CD4^+CD25^+$ T regs as a functionally mature T cell subpopulation.

Sakaguchi and colleagues demonstrated that thymectomy in 3 days old mice led to spontaneous development of autoimmune diseases including gastritis, thyroiditis, and oophoritis (Asano et al., 1996; Sakaguchi et al., 1982; Sakaguchi et al., 1985). The development of these pathologies was linked to the fact that $CD4^+$ $CD25^+$ cells do not migrate out of the thymus until after day 3 of life.

Other studies demonstrated that $CD4^+CD25^+$ T cells could develop *in vitro* in foetal thymic organ cultures, and $CD4^+CD25^+$ thymocytes were as effective as peripheral $CD4^+CD25^+$ T cells in mediating suppression *in vitro* (Itoh et al., 1999). Furthermore, transfer of a mature thymocyte suspension depleted of $CD25^+$ thymocytes produced various autoimmune diseases in mice (Itoh et al., 1999). These results indicate that the normal thymus is continuously producing pathogenic self reactive $CD4^+$ T cells as well as functionally mature $CD4^+CD25^+$ Treg capable of controlling them.

It has been recently shown that the thymic ontogeny of $CD4^+CD25^+$ Treg cells occurs in the human foetal thymus throughout the development of conventional T cells during the step of maturation of $CD4^+$ $CD8^+$ $CD3^{high}$ thymocytes (Cupedo et al., 2005; Arrasse-Jeze et al., 2005). $CD4^+CD25^+$ Treg cells could be detected in human foetuses as early as at 13 weeks of gestation, together with the first mature T cells. Interestingly, they exert suppressive function already in the thymus and they can maintain homeostasis within the developing fetus (Cupedo et al., 2005). Accumulating evidence also indicates that thymic

development of $CD4^+CD25^+$ Tregs require unique interactions of their TCR with self-peptide/MHC complexes expressed by thymic stromal cells (Jordan et al., 2001).

Furthermore, compared with thymic selection of other T cells, the development of $CD4^+CD25^+$ Tregs requires higher-avidity interactions of their TCRs with self peptide/MHC or class II MHC itself expressed on the thymic stromal cells (especially cortical epithelial cells), yet the required avidity must not be so high as to lead to their deletion (Apostolou et al., 2002; Jordan et al., 2001).

1.3.2 Phenotype of $CD4^+$ T regulatory cells

Naturally occurring $CD4^+$ Tregs constitutively express a variety of cell surface molecules more commonly associated with activated/memory cells, most significantly CD25, $CD45RB^{low}$, CD62L, CD103, cytotoxic T lymphocyte antigen-4 (CTLA-4, or CD152), and glucocorticoid-induced TNF receptor family–related gene (GITR) (Powrie et al., 1994; Read et al., 2000; Sakaguchi et al., 1995; Shimizu et al., 2002).

Even though none of these markers is uniquely expressed by naturally occurring $CD4^+$ Tregs, their level of expression and constitutive nature have still made them useful as functional descriptors by enabling the consistent isolation and investigation of $CD4^+$ T cells with regulatory properties.

The transcription factor FOXP3 (or Foxp3 in mouse) seems to be a "master control gene" for their development and function and one of the most used markers for Tregs.

1.3.2.1 CD25

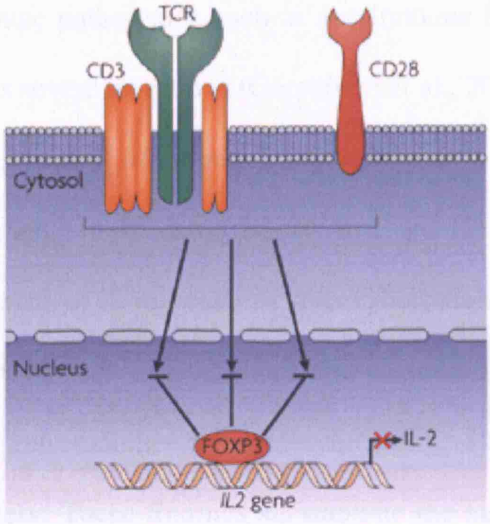
Although CD25 has so far proven to be the best surface marker for thymically produced $CD4^+$ Tregs, it can be expressed on any T cell following activation (Sakaguchi, 2004). In the human system, where there are relatively large numbers of activated T cells, this is especially problematic. Currently, therefore, the best way to select natural human $CD4^+$ Tregs is to sort the population that is very high in CD25. Human $CD25^+$ cells can be split into suppressive ($CD25^{high}$) and non suppressive ($CD25^{low}$) T cells according to the level of expression of CD25 (Baecher-Allan et al., 2001).

Several lines of evidence indicate that CD25 is indispensable for the maintenance of natural $CD4^+CD25^+$ Tregs in the immune system. For example, it has been shown that mice deficient in IL-2, IL-2Rα (CD25), or IL-2Rβ (CD122) develop lethal inflammatory disease, termed IL-2 deficiency syndrome, which can be prevented by inoculation of normal $CD4^+CD25^+$T cells as long as a source of IL-2 is made available experimentally (Almeida et al., 2002; Malek et al., 2002). Recent studies indicated that neutralization of IL-2 selectively reduced numbers of $CD4^+$ $CD25^+$ T cells in normal mice and consequently produced organ-specific autoimmune diseases similar to those produced by depletion of natural Tregs (Setoguchi et al., 2005). Collectively, these results suggest that IL-2 is essential for the development, maintenance, and function of $CD4^+CD25^+$Tregs. Targeting IL-2 can therefore be a possibility to modulate Tregs.

1.3.2.2 FOXP3

The Treg specific transcription factor FOXP3 is a member of the forkhead/winged-helix family of transcription factors. These transcription factors control a wide range of biological processes, including lineage commitment and developmental differentiation (Ramsdell, 2003).

$CD4^+CD25^+$ peripheral T cells and $CD4^+CD25^+CD8^-$ thymocytes specifically express FOXP3, whereas other thymocytes, T cells, B cells, natural killer cells and NKT cells do not (Fontenot et al., 2003; Hori et al., 2003). Notably, in contrast to the stable expression of FOXP3 in natural Treg cells, activated naive T cells or differentiated T helper type 1 or type 2 cells do not express FOXP3, indicating that its expression is highly specific for natural Treg cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Foxp3-deficient mice fail to develop $CD25^+CD4^+$ Treg cells and succumb to scurfy-like inflammatory diseases, which can be prevented by transfer of normal $CD25^+CD4^+$ Treg cells (Fontenot et al., 2003). Furthermore, retroviral transduction or transgenic expression of Foxp3 in $CD25^-CD4^+$ T cells or $CD8^+$ T cells phenotypically and functionally converts them to natural Treg -like cells; for example, Foxp3-transduced $CD25^-CD4^+$ T cells are able to suppress proliferation of other T cells *in vitro* as well as suppress the development of autoimmune disease and inflammatory bowel disease *in vivo* (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Transduction of Foxp3 also suppresses IL-2 production but up-regulates the expression of Treg cell associated molecules, such as CD25, cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and glucocorticoid-inducible tumour necrosis factor receptor (GITR).



Campbell DJ, *Nature Rev Immunol*, 2007.

Figure 1.5 - A model of direct regulation of TCR-mediated signaling by FOXP3.
In the mechanisms of tolerance that control and regulate effective T cell activation, FOXP3 blocks TCR signaling through the inhibition of transcriptional activation mediated by NFAT, NF-κB and AP1 and blocks IL-2 transcription gene.

The identification of mutations in the gene encoding FOXP3 as the cause of the fatal human autoimmune disorder 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX) and the analogous disease in a spontaneous mutant mouse, scurfy, was a breakthrough in the field and led to subsequent studies that argue for the idea of T reg cells as a dedicated functional lineage (Sakaguchi, 2005).

At a very young age, human patients with this autoimmune syndrome present with massive lymphoproliferation, early-onset insulin dependent diabetes mellitus, thyroiditis, eczema, severe enteropathy and food allergies preventing normal food intake, and additional

autoimmune pathologies such as autoimmune haemolytic anaemia and thrombocytopenia, as well as severe infections (Gambineri et al., 2003).

The development of natural T reg cells is at least in part genetically and developmentally programmed. It is most likely that in IPEX, disruption of FOXP3 abrogates the development of T reg cells or alters their function, leading to hyperactivation of T cells reactive with self antigens, intestinal bacteria or innocuous environmental substances, thus causing autoimmune polyendocrinopathy, inflammatory bowel disease and allergy, respectively. These findings substantiate the key contribution of natural T reg cells to the control of immune responses to both self and non-self antigens in humans. Notably, females with a hemizygous defect in FOXP3, which produces genetic mosaicism of normal and defective T reg cells because of random inactivation of the X chromosome in individual T reg cells, are completely normal and do not have an intermediate disease phenotype (Tommasini et al., 2002). This indicates that the residual normal T reg cells dominantly control self-reactive T cells in these females, similar to $CD4^+CD25^+$ T reg cell replenished scurfy mice, demonstrating that the mechanism of dominant self-tolerance is operating physiologically in humans.

Similar sequelae are found in scurfy mutant and Foxp3-deficient mice, including severe dermatitis, aggressive lymphoproliferation resulting in gross enlargement of secondary lymphoid organs, lymphocytic infiltration of multiple organs, hypergammaglobulinemia and autoimmune haemolytic anaemia. Analysis of the scurfy mutant before the identification of the causative mutation demonstrated that the disease is mediated by T cells, with $CD4^+$ T cells being the primary effectors of the disease (Blair et al., 1994; Godfrey et al., 1991; Godfrey et al., 1994).

1.3.3 Functional characteristics of $CD4^+CD25^+$ T regulatory cells and mechanism of action

The ability of $CD4^+CD25^+$ cells to inhibit a range of different immune responses suggests that they function through several different mechanisms (Maloy and Powrie, 2001; Sakaguchi et al., 2001).

Suppression of T cell responses *in vitro* appears to be cytokine-independent and involve unidentified direct interactions between cells. The suppressive function of these cells needs a cell-cell interaction as demonstrated by transwell experiments where T reg were not able to suppress proliferation of T eff cells when not in contact with each other (Jonuleit et al., 2002).

$CD4^+$ $CD25^+$ Tregs themselves are anergic *in vitro*, i.e., they do not proliferate or produce IL-2 in response to conventional T cell stimuli such as plate-or bead–bound anti-CD3, concanavalin A (ConA), or splenic APCs. This anergy can, however, be broken by a sufficiently potent stimulus, e.g., the addition of high-dose exogenous IL-2 , anti-CD28, or IL-15 or the use of mature DCs as APCs (Thornton et al., 2004; Thornton and Shevach, 1998; Yamazaki et al., 2003). Some of these strong stimuli, particularly mature DCs, also perturb $CD4^+CD25^+$ Treg suppression both *in vitro* and *in vivo* (Takahashi et al., 1998; Yamazaki et al., 2003). At least *in vitro*, anergy seems to be the default state of naturally occurring Tregs, since they revert back to it once potent stimulation is withdrawn (Takahashi et al., 1998). *In vivo*, however, $CD4^+CD25^+$ Treg anergy is not readily observed; instead they seem to have a highly active rate of turnover (Almeida et al., 2002; Gavin et al., 2002). It seems likely, then, that $CD4^+CD25^+$ Treg anergy is an *in vitro* phenomenon,

merely reflecting an exacting set of activation requirements generally absent from cell culture.

The mechanism via which $CD4^+CD25^+$ T cells regulate T responses *in vivo* are complex, and several immunosuppressive cytokines such as TGF-β and IL-10, have been implicated in $CD4^+CD25^+$ effector functions.

A number of characteristics have been described for murine $CD4^+CD25^+$ cells that may provide an insight into their mechanism of action.

A number of different mechanisms have been linked to Treg activity, including cell contact–dependent inhibition of the activation and proliferation of antigen-presenting cells (APCs) and T cells, the killing of either APCs or T cells or both, and suppression via cytokines such as IL-10 and TGF-β (Shevach, 2006; von, 2005).

These results suggest that $FOXP3^+$ Treg cells do not suppress immune responses by a single mechanism but use a variety of pathways in a context-dependent manner; for example, depending on cytokine milieu, the activation status of APCs, and the strength of antigen stimulation. A key challenge therefore is to validate putative mechanisms of Treg activity *in vivo* and define the circumstances in which these operate. An important factor may be the site of action of Treg cells.

1.3.3.1 Transforming growth factor-β (TGF-β)

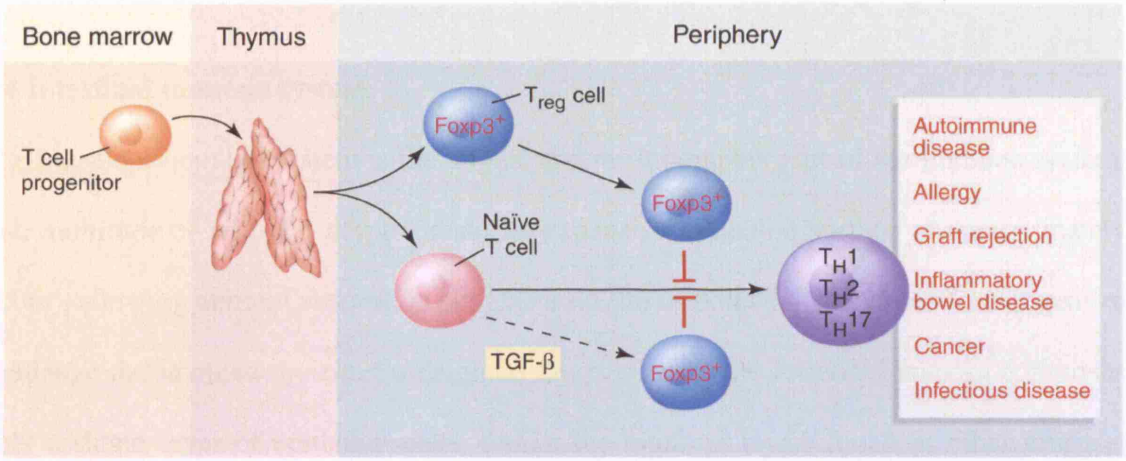
TGF-β appears to play a general role in Treg cell activity, being required for control of autoimmune disease as well as intestinal inflammation (Maloy and Powrie, 2001). T cells are a key target of TGF-β, as $CD4^+$ or $CD8^+$ T cells expressing a dominant-negative TGF-β receptor, and therefore cannot respond to TGF-β, escape control by $CD4^+CD25^+$ T cells *in vivo* (Green et al., 2003). TGF-β might act directly on potentially pathogenic T cells to

inhibit their differentiation and effector function (Gorelik and Flavell, 2002). Some $CD4^+CD25^+$ T reg cells express membrane-bound TGF-β, although there are conflicting data on its functional role (Nakamura et al., 2001; Piccirillo et al., 2002). Recent studies suggest that autocrine production of TGF-β1 by T reg cells is essential for their function, as TGF-$\beta1^{-/-}$ $CD4^+CD25^+$ T reg cells failed to suppress colitis in mice (Nakamura et al., 2004).

1.3.3.2 IL-10

Like the TGF-β results, the data for IL-10 are contradictory. IL-10 is expressed by various immune cells, including T cells, B cells, and macrophages. Its wide range of immunosuppressive effects include decreased T cell cytokine production, decreased antigen presentation, and decreased expression of co-stimulatory molecules on APCs. Mice lacking IL-10 develop a spontaneous enterocolitis. Isolated $CD4^+CD25^+$ cells will produce IL-10, but this activity does not seem necessary for *in vitro* suppression (Asano et al., 1996; Takahashi et al., 1998). However, *in vivo* IL-10 is necessary for suppression of autoimmune colitis but not gastritis (Suri-Payer and Cantor, 2001).

There is evidence that Treg cells act in tissues to control established inflammation and that Treg cell production of IL-10 plays a functional role (Maloy and Powrie, 2001). IL-10–secreting $Foxp3^+$ T cells are rare in the spleen but abundant in the inflamed intestine and also become detectable at the site of inflammation in autoimmune disease or chronic infection (Uhlig et al., 2006). This indicates that there is compartmentalization of the Treg response and that the mechanisms of suppression may be influenced by the anatomical location and dictated by the nature of the inflammatory response being regulated.



Sakaguchi S and Powrie F, *Science*, 2007

Figure 1.6 - Origins of Treg cells and the expression of the transcription factor FOXP3. FOXP3$^+$ natural Treg cells produced by the normal thymus suppress the activation and expansion of naïve T cells and their differentiation to effector T cells, including Th1, Th2, and Th17 cells, which mediate a variety of pathological and physiological immune responses. FOXP3$^+$ Treg cells can also be generated from naïve T cells in the periphery.

1.3.4 Induction of CD4$^+$CD25$^+$ Tregs

It is well accepted that CD4$^+$ T cells with regulatory function can be generated by the activation of mature, peripheral CD4$^+$CD25$^-$T cells, and both regulatory and pathogenic T cells can, in principle, be generated from the same mature T cell precursors, depending on qualitative and/or quantitative differences in antigen priming. These induced Treg cells (iTreg) can be generated *ex vivo* from mature CD4$^+$CD25$^-$T cell populations under different stimulatory conditions including, antigen in the presence of immunosuppressive cytokines, such as IL-10 and TGF-β1, vitamin D3 and dexamethasone, CD40-CD40L blockade or immature DC populations (Barrat et al., 2002; Zheng et al., 2002). It must be noted that iTreg cells function *in vitro* and *in vivo* generally in a cytokine-dependent manner. Antigen exposure by intranasal, intradermal or oral route seems to selectively induce the appearance of T cells with regulatory phenotype (Bach, 2003; Shevach, 2000).

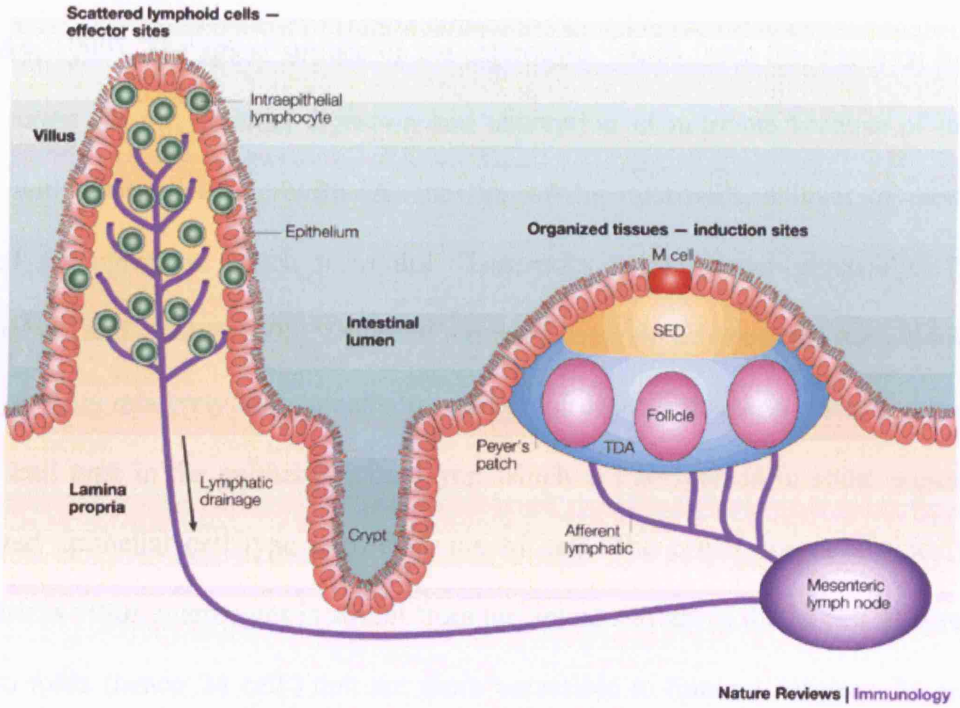
1.4 Intestinal immune system

The intestinal immune system is the largest and most complex part of the immune system. The multitude of villi and crypts create an expansive epithelial surface of approximately $400m^2$, allowing nutrient absorption but also a wealth of potential entry sites for antigens to challenge the immune system. To heighten the challenge, the intestinal mucosa comprises only a single layer of epithelial cells, unlike the multiple layers found at other mucosal surfaces. Most human pathogens enter the body through a mucosal surface, such as the intestine, and strong immune responses are required to protect this physiologically essential tissue. The intestinal immune system encounters more antigen than any other part of the body, but also it must discriminate clearly between invasive organisms and harmless antigens, such as food proteins and commensal bacteria (Mowat, 2003). Epithelial cells are replaced every 2–5 days from pluripotential stem cells in the base of the crypts (Booth and Potten, 2000) and so continuous antimicrobial protection for these stem cells is of paramount importance as damage to or parasitisation of stem cells would have severe consequences for the maintenance of the normal digestive epithelium.

The microbial density in the healthy proximal small intestine (duodenum, jejunum, proximal ileum) is low. This relative paucity of bacteria in the upper tract when compared with the colon seems to be because of the composition of the luminal medium (gastric acid, bile, pancreatic secretion), which kills most ingested micro-organisms, and because of the peristaltic propulsive motor activity which impedes stable colonization of bacteria in the lumen. By contrast, the colon contains a complex and dynamic microbial ecosystem with high densities of living bacteria, which achieve concentrations of up to 10^{11} or 10^{12} cells/g

of luminal contents consisting of ~400 different species of anaerobic and aerobic bacteria (Guarner and Malagelada, 2003). In developed countries, improved standards of sanitation in food preparation have resulted in most infectious diseases of the gut with bacterial pathogens being largely under control. However gastrointestinal food allergies and idiopathic inflammatory conditions have dramatically increased (Macdonald and Monteleone, 2005) in prevalence as standards of sanitation have improved. Although the reason for this remains unknown, a prevailing notion is that because of the huge variety of antigens and the large number of lymphoid cells in the intestine, minor dysfunction of mucosal immune homeostasis may induce an intestinal immune response resulting in inflammation and chronic disease (Monteleone et al., 2002). Healthy individuals possess an abundant and highly active gut immune system that is tightly regulated to prevent excessive immune responses to foods and gut bacteria.

A major difference between the systemic and mucosal immune system is the anatomical separation of the initiation compartment, defined as the organized Gut-associated lymphoid tissue (GALT) from the effector sites in lamina propria and epithelium.



Mowatt AM, *Nature Review Immunology*, 2003

Fig 1.7 - Gut Associated Lymphoid tissue (GALT)
The gut-associated lymphoid tissue (GALT) is constantly exposed to a variety of Ags. When the GALT receives signals from the intestinal flora or food Ags, it must induce a state of nonresponsiveness (mucosal tolerance). In contrast, when pathogenic bacteria invade the intestinal mucosa, it is necessary to elicit strong T and B cell responses. The GALT is therefore in the position of constantly fighting intolerance to food and the commensal flora while effectively battling infectious microbes.

Although consisting of only a single layer of cells, the intestinal epithelium must control the access of potential antigens and pathogens, and, at the same time, function in the digestive absorption of dietary nutrients. It is aided in this dual role by intercellular Tight junctions that restrict the passage of even very small (2-kDa) molecules (Madara, 1998). The intestinal epithelium also boasts a number of specialized protective adaptations not found in other barriers including anti-microbial peptides (defensins) (Ayabe et al., 2000),

secretory immunoglobulin A (Macpherson et al., 2000), mucins and trefoil peptides (Podolsky, 1999). The apical surface of the enterocyte, which faces the intestinal lumen, is ideally suited for the terminal digestion and absorption of nutrients because of its dense coating with absorptive microvilli. At the tips of the microvilli, a layer of membrane-anchored glycoproteins which form the filamentous brush border glycocalyx (FBBG) (Kraehenbuhl and Neutra, 2000). Digested nutrients can gain access to the body through the FBBG, but it is relatively impermeable to macromolecules or bacteria. Enterocytes are the primary cell type in the epithelial monolayer, which is interspersed in some regions by a specialized epithelial cell type known as the M cell. The brush border glycocalyx that characterizes villus enterocytes is absent from the apical surface of the M cell. It is replaced by micro folds (hence 'M cell') that are more accessible to luminal antigens. M cells use trans-epithelial vesicular transport to carry microbes to APCs in the underlying gut-associated lymphoid tissue (GALT). The GALT is the site where antigen presentation occurs. The GALT can be divided into effector sites, which consist of lymphocytes, scattered throughout the epithelium and lamina propria of the mucosa, and organized tissues that are responsible for the induction phase of the immune response. These are the Peyer`s patches (PP) and mesenteric lymph nodes (MLN), as well as smaller, isolated lymphoid follicles, which have the appearance of microscopic PP and are distributed throughout the wall of the small and large intestines (Hamada et al., 2002). The PP are macroscopic lymphoid aggregates that are found in the sub-mucosa along the length of the small intestine. Mature PP consist of collections of large B-cell follicles and intervening T-cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated epithelium (FAE), and a more

diffuse area immediately below the epithelium, known as the sub-epithelial dome (SED) (Mowat, 2003). The FAE differs from the epithelium that covers the villus mucosa, as it has lower levels of digestive enzymes and a less pronounced brush border also it is infiltrated by large numbers of B cells, T cells, macrophages and dendritic cells (DCs) and has micro fold (M) cells. The MLNs are the largest mass of lymph nodes in the body. Accumulation of lymphocytes in the MLNs requires both L-selectin and $\alpha_4\beta_7$ adhesion molecules, which normally direct lymphocytes to enter peripheral and mucosal tissues, respectively (Wagner et al., 1998). As a result of these unique anatomical features, the MLNs might be a crossroads between the peripheral and mucosal recirculation pathways. It has been assumed for many years that M cells provide the main, if not the only, way in which complex antigens can gain access to the intestinal immune system. M cells probably do not process antigens themselves — they do not express MHC class II molecules — and instead, they are believed to pass on intact antigen to professional APCs, either in the epithelium or in the underlying dome region. From there, the APCs move to the T-cell areas and/or B-cell follicles, where they can interact with naive lymphocytes. DCs are probably the APC involved in this process, and several DC subsets have been described recently in PP. In PP, B cells undergo immunoglobulin class switching from expression of IgM to IgA under the influence of several local factors, including transforming growth factor-β (TGF-β) and IL-10. The lymphocytes that are primed in the PP exit through the draining lymphatics to the MLNs, where they reside for an undefined period of further differentiation, before they migrate into the bloodstream through the thoracic duct and finally accumulate in the mucosa. The exit of lymphocytes into the mucosa occurs because lymphocytes that are primed by antigen in the GALT lose expression of L-selectin and

selectively up-regulate expression of integrin $\alpha_4\beta_7$. This directs the emigration of lymphocytes from the bloodstream by interacting with the ligand for $\alpha_4\beta_7$ integrin, mucosal addressing cell-adhesion molecule 1 (MADCAM1), which is expressed at high levels by the vasculature of mucosal surfaces (Berlin et al., 1993; Butcher et al., 1999). In parallel, expression of the chemokine receptor CCR9 is induced by gut-derived T cells, allowing them to respond to the chemokine CCL25, also known as TECK (thymus-expressed chemokine), which is expressed selectively by small-bowel epithelial cells (Bowman et al., 2002; Campbell and Butcher, 2002). This pattern of adhesion-molecule and chemokine-receptor expression is distinct from that of T cells that are primed in peripheral lymphoid organs, which acquire the $\alpha_4\beta_1$ integrin VLA4 (very late antigen 4) and the chemokine receptor CCR4 and so cannot migrate to mucosal surfaces (Campbell and Butcher, 2002). The main effector compartment consists of the Lamina Propria (LP), to which numerous antigen-experienced T cells migrate after stimulation in the initiation compartment (Brandtzaeg and Pabst, 2004). The LP is filled with antibody-producing plasma cells that secrete IgA immunoglobulin into the gut lumen that function mainly as an inhibitor of bacterial or viral attachment to the underlying epithelium (Macpherson et al., 2001). It has been also identified that IgA immunoglobulins are able to recognize the autoantigen tissue transglutaminase (tTG) in the mucosa of celiac patients (Dieterich et al., 1997). Numerous other immune cells also reside in the gut LP, including large numbers of $CD4^+$ T cells, macrophages, DC, mast cells, and eosinophils. Some of these antigen-experienced lamina-propria T cells might be true effector cells, and might help local B cells to produce IgA. $CD4^+$ T cells in the lamina propria are of particular importance to local immune regulation. They might be regulatory T cells and therefore responsible for

maintaining local tolerance to environmental antigens (Khoo et al., 1997). The gut epithelium also contains abundant intraepithelial lymphocytes (IEL) (Hayday et al., 2001), an intriguing population made up mostly of $CD8^+$ T cells. Compared with other tissues, IEL are enriched in T cells expressing the γδ T cell receptor (Cheroutre, 2004). The exact function of IEL is not known, although it has been suggested that they may play a role in epithelial tumour surveillance, protection against epithelial pathogens, or promotion of healing of the gut after injury (Hayday et al., 2001).

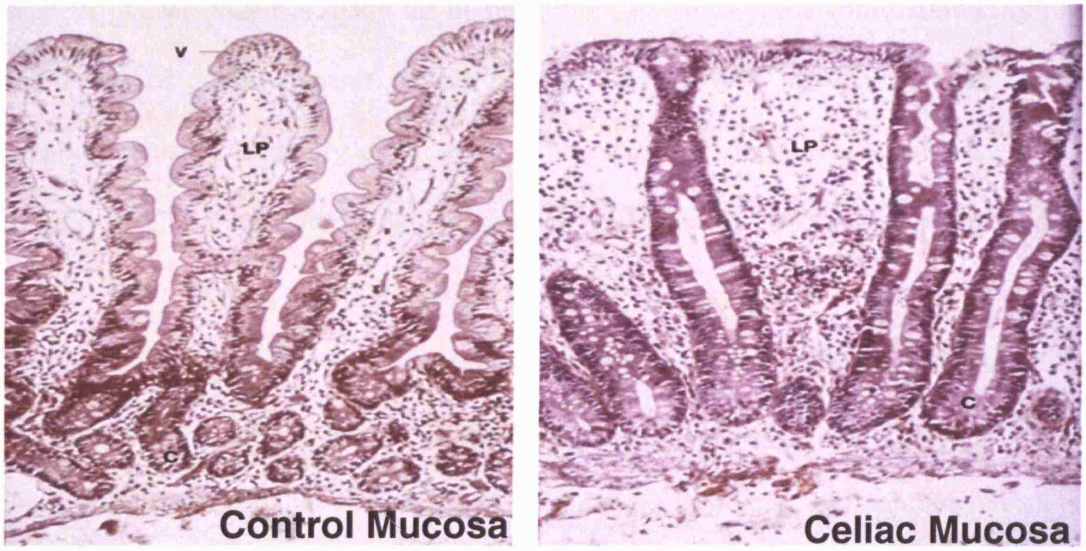
The presence of an extensive and activated intestinal immune system depends on the commensal flora. It has been noted that germ-free animals are relatively immunodeficient (Mayer, 2003). Thus, mice bred under germ-free conditions possess small, underdeveloped PP lacking germinal centres, few IgA plasma cells and CD4 cells in the LP, and reduced numbers of IEL. Furthermore, reconstitution of germ-free mice with a microbial flora is sufficient to restore the mucosal immune system (Umesaki et al., 1995). Therefore the relationship between the immune system and the commensal flora is a precarious one, and perturbations in immune or epithelial homeostasis can lead to gut inflammation including Celiac Disease (CD) and chronic inflammatory bowel disease (IBD).

1.5 Celiac Disease

The development of agriculture, which started in the Middle East about 10,000 years ago, not only led to the development of ancient civilizations but also resulted in radical changes in the composition of the human diet. One of those changes was the introduction of cereal-based food products and today such food products are very common in a normal diet. Yet approximately 1% of the population in the developed world cannot tolerate cereals and

suffers from Celiac disease (CD) (Fasano et al., 2003; Maki et al., 2003). CD is most likely as old as cereal consumption and its symptoms were described by the Roman physician Galen. But it was not until the 1950s that gluten, the grain storage proteins, was found to be responsible for the occurrence of the clinical manifestations in CD patients. More recently, the role of HLA in the development of an inflammatory T cell response to the gluten has been elucidated. It is still unclear, however, why only a minority of predisposed individuals actually develop CD. CD is a complex autoimmune enteropathy and it is characterized by small-intestinal mucosal injury which may lead to nutrient malabsorption. CD can present with a wide range of symptoms, varying from "classical" CD through atypical presentations to "silent" CD. Classic presenting symptoms and signs of CD include abdominal pain, diarrhoea, bloating, fatigue, nausea, vomiting, weight loss, and anaemia. These typically appear in early childhood, but occasionally present in later life. The severe characteristic change, which occurs in the mucosa of the small bowel, is the loss of absorptive villi with a flat mucosal surface accentuated by hyperplasia of the enteric crypts. The normal small intestinal mucosa of healthy individuals is characterized by tall villi lined by a single layer of columnar epithelial cells with nuclei located near the basement membrane, and numbers of intra-epithelial lymphocytes (IEL) approximating to 1 per 6-10 epithelial cells (or enterocytes). The lamina propria contain lymphocytes and plasma cells in numbers consistent with the "physiologic inflammation" that is normal in the small intestine, and the ratio of villous height to crypt depth is approximately 4:1 to 5:1. In contrast, the small-intestinal mucosa of patients with severe CD may show total villous atrophy. The complete loss of villi is accompanied by the presence of markedly abnormal squamoid surface epithelial cells, an increase in the number of IELs, a marked

increase in the number of lymphocytes and plasma cells in the lamina propria, and striking crypt hypertrophy with increased crypt mitoses (Marsh, 1992). There are in fact a range of morphological changes seen in the small intestine that have been classified by Marsh (Marsh, 1992) but all have similar immunopathology.



Sollid LM, *Nature Review Immunology*, 2002

Figure 1.8 Small intestine from control mucosa and from CD mucosa.
In CD mucosa crypt hyperplastic villous atrophy, an abnormal surface epithelium and lamina propria inflammation are observed compared with control mucosa.

Many individuals with CD manifest predominantly extra intestinal symptoms and findings (for example, unexplained iron deficiency anaemia, premature-onset osteoporosis, irritability, and depression) or are relatively asymptomatic (for example, individuals identified only because they have affected family members). CD has many characteristics of a chronic inflammatory disease. Susceptibility to CD, and its activation and perpetuation, involve a combination of environmental and genetic factors, and

immunological mechanisms. It is postulated that CD is the result of an inappropriate T cell-mediated immune response against ingested proteins. It is in fact activated in genetically susceptible individuals by the dietary ingestion of proline- and glutamine-rich proteins that are found in wheat, rye, and barley and are widely termed "gluten" (Kagnoff, 2005), but, strictly speaking, gluten only encompasses the disease-activating proteins in wheat. In wheat gluten is made up of insoluble (glutenin) and alcohol soluble (gliadin) components of the endosperm protein storage fraction, both of which contain disease-activating peptides (Molberg et al., 2003). The closely related proteins in barley and rye that activate CD are the hordeins and secalins, respectively (Vader et al., 2003). Wheat, rye, and barley have a common ancestral origin in the grass family. Oats are thought to activate CD only rarely (rentz-Hansen et al., 2004), and, consistent with this, oat avenins are more distantly related to the analogous proteins in wheat, rye, and barley and have substantially lower proline content. Gliadins, glutenins, hordeins, and secalins have a high proline and glutamine content. The high proline content renders these proteins resistant to complete proteolytic digestion by gastric, pancreatic, and brush border proteases and peptidases in the human intestine, since those enzymes are deficient in prolyl endopeptidase activity (Shan et al., 2002). This can result in the accumulation of relatively large peptide fragments (as many as 50 amino acids in length) with a high proline and glutamine content in the small intestine (Shan et al., 2002). In recent years, the prevalence of CD appears to have increased sharply probably as a result of better recognition of the disease with the introduction of sensitive serological screening. Indeed such screening suggests that the prevalence of CD is approximately 1% or more in the general population (Fasano et al., 2003; Maki et al., 2003). The most sensitive and specific screening methods

involve measurement of an autoantibody. Formerly this was called "anti-endomysial antibody (EMA) when measured by immunofluorescence but now it is called anti-tissue Transglutaminase (tTG) when measured by ELISA (Sulkanen et al., 1998). The sole lifelong therapy for this condition is avoidance of gliadin (the alcohol-soluble protein of wheat gluten) and similar proteins in rye and barley.

1.5.1 Genetic factors in CD: MHC class II and HLA-DQ alleles

Genetic factors are important in the pathogenesis of CD. This was first evident from clinical observations of multiple cases of CD within families, and the high (approximately 70%–75%) rate of concordance for CD among monozygotic twins (Greco et al., 2002). It is known that CD is associated with specific MHC class II alleles that map to the *HLA-DQ* locus (Sollid et al., 1989). The human major histocompatibility complex (MHC) molecules DQ2 and DQ8 are essential genetic factors for the development of celiac disease, with the majority of patients carrying DQ2. In the remaining patients, an association with DQ8 is found (Louka and Sollid, 2003b). These HLA genes confer up to 40% of the genetic risk; the rest is attributable to non-HLA genes (Louka and Sollid, 2003). The HLA-DQ2 heterodimers that confer susceptibility to CD are formed by a β chain encoded by the allele *HLA-DQB1*02* (either *HLA-DQB1*0201* or **0202*) and an α chain encoded by the allele *HLA-DQA1*05*. This HLA-DQ2 heterodimer is present in at least 90%–95% of patients with CD (Sollid and Lie, 2005), although a very small number of CD patients have been reported in whom only one of these DQ2 alleles is present (that is, *HLA-DQB1*0202* or, rarely, *HLA-DQA1*05*) (Sollid and Lie, 2005). The HLA-DQ8 heterodimer found in the remaining 5%–10% of patients with CD is formed by the β chain and α chain encoded by *HLA-DQB1*0302* and *HLA-DQA1*03*, respectively (Sollid and Lie, 2005). The *HLA-DQ2*

alleles associated with increased susceptibility to CD can be inherited in *cis* (that is, on one parental chromosome) or in *trans*, with the HLA-DQ alleles forming the HLA-DQ2 heterodimer being encoded on one chromosome from each parent (Sollid et al., 1989). Notably, CD is substantially more prevalent in those in whom 100% or approximately 50% of the HLA-DQ heterodimers are HLA-DQ2 than in those in whom only approximately 25% of the HLA-DQ heterodimers are HLA-DQ2 (Ploski et al., 1993).

1.5.2 Adaptive immune response in CD

The strong association of HLA-DQ2 and –DQ8 with CD prompted the search for components of gluten that could be presented by MHC class II. Gluten is a common, imprecise term for disease-activating proteins in wheat, barley and rye.

Three key pieces of work underpin the current understanding of the role of HLA-DQ2 and the adaptive immune system in CD. In 1993 Lundin et al. (Lundin et al., 1993), isolated $CD4^+CD25^+$ T lymphocytes from small intestinal biopsies of $HLA\text{-}DQ2^+$ adult celiac patients. These cell lines responded by proliferation to stimulation with fragments of the gliadin component of gluten, but this response could be inhibited by treatment with anti-HLA-DQ2 antibodies. This demonstrated that T cells responded to the alcohol-soluble fraction of wheat storage protein (gliadin) and that blocking HLA-DQ2 abrogated the T-lymphocyte response. The obvious and exciting implication of this work was that gliadin-derived peptides were stimulating T cells via presentation by HLA-DQ2. To address this possibility Johansen et al. (Johansen et al., 1996), tested overlapping peptides derived from gliadin in binding and T-cell stimulation assays. Disappointingly this showed only weak binding of gliadin peptides to HLA-DQ2. In 1998 however Van de Wal et al.(van de Wal et al., 1998), noted that the HLA-DQ2 peptide-binding motif demonstrated a preference for

negatively charged residues at several positions. They also observed that auto-antibodies to tissue Transglutaminase (tTG) are commonly seen in CD (indeed today this forms the basis for serological diagnosis) and that this enzyme could result in the deamidation of the plentiful Glutamine (Q) residues in gliadin to negatively charged Glutamic acid (E). They showed that the presence of tTG in their *ex vivo* assays increased mucosal gliadin-specific T-cell recognition. This elegant study was further refined by Arentz-Hansen (Arentz-Hansen et al., 2000), who showed, by screening overlapping recombinant peptide libraries of gliadin, that *ex vivo*, derived T cells cloned from all the celiac patients tested in the study, recognized a single deamidated glutamine residue with the gliadin sequence. This gliadin-derived peptide PFPQPQLPY both bound HLA-DQ2 and stimulated T cells poorly but after tTG deamidation to PFPQPELPY bound to HLA-DQ2 with 25 times higher affinity and was extremely immunogenic.

1.5.3 Role of tissue Transglutaminase in CD

Tissue Transglutaminase (tTG) is a Ca^{2+}-dependent enzyme that is responsible for the post-translational modification of proteins by transamidation or deamidation of specific polypeptide-bound glutamines. tTG catalyses the deamidation of specific glutamine residues to glutamic acid, producing the negatively charged residues necessary for efficient binding to DQ2 and DQ8 and for T cell activation (Molberg et al., 1998). The spacing between the targeted glutamine and neighbouring proline residues is particularly important for enzyme specificity.

CD patients on a gluten-containing diet produce immunoglobulin IgA and IgG autoantibodies that are specific for tTG. tTG may have a further role in CD not only as the target autoantigen or as the catalytic factor for gliadin pathogenic epitope generation but

also as the regulator of lymphocyte migration and controller of the early epithelial phases of celiac disease (Maiuri et al., 2005). These studies demonstrate that tTG on the luminal side of the surface epithelium (stTG) controls the epithelial phase of gliadin challenge both on isolated epithelial cell lines and in biopsy specimens of celiac patients. In addition it appears that stTG controls cell modifications leading to epithelial changes like actin reorganization, phosphorylation and apoptosis induced by one of gluten peptides.

1.5.4 Innate immune response in CD

HLA is a necessary but not sufficient genetic factor for the development of CD, indicating that the gluten-reactive $CD4^+$ T cells in the lamina propria must control the immune reaction leading to CD (Sollid, 2002). The key to explaining the presence of gluten antigens in the lamina propria, and the initiation of the HLA-DQ2 and/or –DQ8-restricted adaptive immune response, is then to identify the trigger for the loss of the epithelial integrity. Howdle developed an *in vitro* assay (Howdle et al., 1981) to assess changes in morphology of small intestinal mucosa in culture with and without gluten challenge. They were able to show significant changes of enterocyte height in co-culture with gluten (compared to culture without gluten) in biopsy samples from treated and untreated CD patients but not from normal controls. In 1994 Sturgess et al. mapped the specific region of gliadin responsible for this rapid villous atrophy (Sturgess et al., 1994). This "toxic" epitope was localized within p31-49 of α-gliadin (the immunogenic or immunodominant epitope binding to HLA-DQ2 is localized within the p56-75 region of α-gliadin). Infusion of 200 mg of this p31-49 peptide, but not two other candidate peptide regions, into four patients with CD resulted in characteristic histological changes within six hour of enteral infusion (assayed by jejunal biopsy). The Sturgess and Howle studies showed that an initial toxic

insult to gut epithelium may be delivered by a region of gliadin not recognized by the adaptive immune system rather than any external event. Further studies, showed that this toxic p31-49 peptide in fact induced the expression of the non-conventional HLA class I molecule MICA on the cell surface of villous epithelium enterocytes in treated CD patients but not normal controls (Hue et al., 2004). They further showed that untreated CD patients already had high level of MICA surface expression and that the surface levels appeared to correlate with the clinical severity of disease. MICA is induced by cellular stress (e.g., tumours, intracellular pathogens) and serves as a ligand for NKG2D receptor. The NKG2D receptor is found on the surface of $CD8^{+}\alpha\beta$ T cells, $\gamma\delta$ T cells and most NK cells (Bahram et al., 2005).

$CD8^{+}$ cytotoxic intraepithelial T cells in the small intestine of CD patients express the NKG2D receptor and at least a subgroup express CD94 (Maiuri et al., 2001) and are capable of killing the enterocytes lining the intestine through the NKG2D-MICA interaction. It has been hypothesized that the intraepithelial T cells could induce epithelial death via the engagement of FAS, the foremost death receptor which is known to be expressed by epithelial cells (Maiuri et al., 2001). Other studies have also shown that gliadin can prompt the killing of the enterocytes by inducing the cytokine IL-15. IL-15 is implicated in the migration and the expansion of intraepithelial lymphocytes (IEL), which are abnormally increased in CD (Maiuri et al., 2001). Also IL-15 in turn induces the expression of the ligand MICA on the enterocytes and the expression of the receptor for this ligand, NKG2D, on the intraepithelial T cells of the intestine (Hue et al., 2004; Meresse et al., 2004).

Previous studies have shown the expression of IL-15, a typical cytokine of the innate immune system, by lamina propria dendritic cells and macrophages in CD patients but not normal controls in response to stimulation with the non-immunodominant p31-49 α-gliadin peptide (Maiuri et al., 2000; Maiuri et al., 2003).

The epithelial changes induced by these peptides in CD activate an innate immune response, mediated by the rapid expression of IL-15 that induces DC activation preparing for the adaptive immune response to the peptides deamidated and presented to $CD4^+$ cells (Maiuri et al., 2003). Inhibition of IL-15 controls such activity, confirming the key role of this innate immune cytokine, as mediator of intestinal mucosa damage induced by ingestion of gliadin.

It appears therefore that epithelial cells, as first barrier in gut mucosa, have a central role in the innate immune response to gluten in CD.

Besides activation and presentation of molecules able to activate neighbouring $CD8^+$ T cells, enterocytes go through cellular modifications induced by intracellular signalling pathways to respond to extracellular stress.

A series of rapid modifications, and morphological changes, at the epithelium level, such as intestinal villus atrophy, decreased enterocyte height and increased intestinal permeability (Cooper, 1983) are induced in CD mucosa by gliadin challenge. These early changes are induced by fragments of gliadin not recognized by the adaptive immune system, the non-immunodominant p31-49 gliadin peptide that is capable to activate an innate immune response (Maiuri et al., 2003; Maiuri et al., 2005).

This innate response is particularly important for mucosal surfaces that are continuously exposed to external pathogens or potential toxins such as the intestine.

All these epithelial changes suggest that the cytoskeleton may be involved in the mechanism of epithelial cell damage observed in CD. In fact one of the early changes, after gluten challenge, observed in the enterocytes of mucosa of celiac patients, is the reorganization of the actin filaments, with induction of stress fibers (Bailey et al., 1989; Holmgren et al., 1995).

1.6 The cytoskeleton and the communication with the external cellular matrix

The cytoskeletal structures formed by proteins permit immediate communication between external cellular stimuli and the cytoplasm. Polymerized, these proteins generate a semi-rigid structure. When a tensile force is applied to one end of the cell, it will twist a number of the fibers, having an impact on the entire cell structure.

The cytoskeleton of vertebrate cells is formed by three different types of filament systems: microfilaments, microtubules and intermediate filaments. Microfilaments are formed by actin subunits, microtubules are formed by tubulin filaments, whereas intermediate filaments are formed from different proteins in a cell-type specific fashion, such as desmin, vimentin and keratin. Microfilaments are, together with actin-binding proteins, essential for cell movement and cell shape reorganization, microtubules are essential for intracellular trafficking as well as cytokinesis, and finally, the intermediate filaments provide cells with mechanical strength (Ku et al., 1999).

Several cell types develop a polarized architecture that is essential for their biological function. Typical polarized cells are epithelial cells of the intestine. In epithelial cells, the apical surface faces the lumen and is the site of secretion or absorption. The basolateral surfaces refer to basal areas, which contain hemidesmosomes and interact with the

extracellular matrix (ECM), and the lateral sides, which interconnect neighbouring cells via gap junctions and desmosomes.

The microfilament system, also known as the actin cytoskeleton, is a highly dynamic structure, which is under a continuous reconstruction to control the morphology, survival, growth and mobility of eukaryotic cells (Pollard et al., 2001).

Actin, is an ATP-binding protein that exists in two forms in the cell, as monomers (globular actin, G-actin), or as filaments (filamentous actin, F-actin).

Actin monomers polymerise by a strictly regulated process into helical polar filaments (Schmidt and Hall, 1998). The two ends of the actin filament have distinct features, the fast growing end, also called the barbed end and the slow growing end, also called the pointed end. The dynamic reconstruction of actin filaments is in turn regulated by actin-binding proteins, such as myosin, tropomyosin, filamin, fimbrin, α-actinin, gelsolin, villin and profilin. Movement of the cell depends on actin specific formations such as lamellipodia, formed at the leading edge of a crawling cell, with thin, flat, sheet-like structures of actin filaments; filopodia are thin protrusions, of tightly packed parallel bundles of actin filaments, extending out from the leading edge and stress fibers, another type of actin filament-containing structures, that consist of bundles of actin filaments and myosin-II filaments. The ends of the stress fibers are attached to the plasma membrane not uniformly, but rather at certain special sites called focal contacts or focal adhesions, which are in association with the extracellular matrix. It is known that these points of contact are important sites for inducing cell activation.

The main transmembrane linker proteins of the interaction between focal contacts, external forces and the cytoskeleton are members of the integrin family. This family of receptors is

composed of a series of heterodimers, formed from one α and one β chain. The different combinations of chains confer specificity, since each heterodimer binds to one or at most to a small number of extracellular matrix (ECM) proteins. Once the integrins bind to ECM, the cytoplasmic portion of the receptor is connected through talin, vinculin, and α-actinin (in series) to loosely bundled, parallel actin filaments that form stress fibers. These polymer networks thus serve as a link through the cytoplasm among various integrin receptors around the cell surface, permitting signals delivered to the external cell membrane to have an impact throughout the cell.

As described above, the integrin receptor is linked directly to the internal structures of the cell in a way that, through a combination of chemical and tensile forces, directs cell function. The focal adhesions are connected by talin, vinculin, and α-actinin to the actin stress fibers, completing the cell structure. Since the adhesions are focal and not uniform, some relocation of cytoskeletal structures must occur as binding takes place, likely introducing tensile forces on the cell. Furthermore, it is clear that focal contacts are important in signal transduction induced by other molecular mediators, such as growth factors and cytokines.

In cells, actin filament assembly depends on the presence of free barbed ends that act as templates for new polymerization. The creation of free barbed ends, which occurs in the initial phase of actin polymerization, involves three general mechanisms: uncapping of pre-existing filaments, severing of filaments, and *de novo* nucleation (refers to the initiation of actin polymerization from free monomers) (Welch and Mullins, 2002). The specific contribution of each mechanism may be cell type specific, but most often all three

mechanisms are involved. The actin polymerization is tightly regulated by transmembrane signalling.

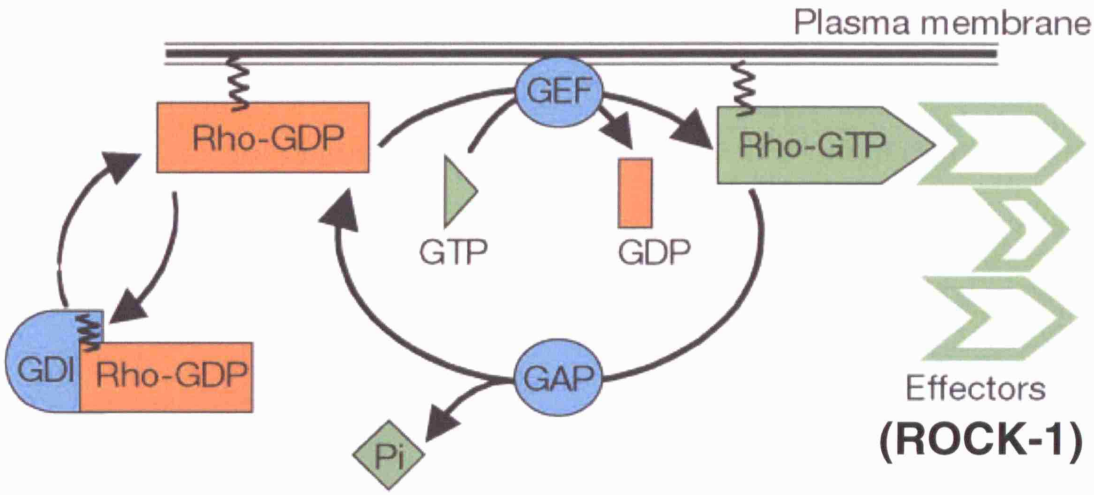
1.6.1 Rho GTPases

Regulation of most actin-dependent processes has been demonstrated to be mediated by Rho family GTPases. Rho GTPases are found in all eukaryotic organisms and regulate cell polarity and motility through their effects on the cytoskeleton, membrane trafficking and cell adhesion (Jaffe and Hall, 2005; Jiang and Ramachandran, 2006). They are present in many different cell types, including epithelial cells (Hall, 1998). Rho family GTPases are members of the Ras gene superfamily of p21 GTPases that function as molecular switches by cycling between GTP and GDP bound states (Hall, 1998). Activity of Rho proteins is regulated by an upstream pathway in response to growth factors and cytokine signalling, and their activity integrates control of a downstream pathway that affects cell morphology and motility, transcription of specific genes, cell cycle progression and apoptosis (Coleman and Olson, 2002; Ridley, 2001). In mammals there are 22 Rho GTPases but the best-studied members of the family are RhoA, Rac1 and Cdc42. These three members of the Rho family are widely expressed and have been shown to induce morphological changes associated with actin polymerization that generates forces that allow movement within the actin skeleton.

As observed in a study on Swiss 3T3 fibroblasts, Rac1 induces membrane ruffling and lamellipodia formation, RhoA induces the formation of stress fibers, and Cdc42 induces the formation of microspikes and filopodia, all of which are dependent on filamentous actin (F-actin) organization (Nobes and Hall, 1995).

1.6.2 The Rho GTPase cycle.

Activation of the GTPase, through GDP-GTP exchange is promoted by guanine nucleotide exchange factors (GEFs), whereas inactivation (by an intrinsic GTPase activity) is stimulated by GTPase-activating proteins (GAPs). Rho guanine nucleotide dissociation inhibitors (Rho-GDIs) appear to stabilize the inactive, GDP-bound form of the protein **(Figure 1.9)**. Activated Rho GTPases interact with cellular target proteins or effectors to trigger a wide variety of cellular responses, including the reorganization of the actin cytoskeleton and changes in gene transcription. Rho GTPases exist in an inactive GDP-bound state complexed to GDI. In response to an incoming signal, Rho-GDI dissociates, and GEF catalyse nucleotide exchange stimulating release of GDP and allowing GTP to bind. This occurs at or close to the plasma membrane. In this active state, the GTPase interacts with cellular targets or effectors to generate a cellular response. Most of the targets are ubiquitously expressed, and it unknown how a GTPase "chooses" its target under a particular set of circumstances. Rho GTPases can, in their GTP-bound state, interact with numerous target proteins to induce coordinated signals. Finally, Rho GAPs will interact with the GTPase-target complex and catalyze GTP hydrolysis (Van and Souza-Schorey, 1997), converting the proteins to the GDP-bound inactive conformation. The GDP-bound form of the GTPase is then "extracted" from the membrane by Rho-GDI, and the cycle is complete (Etienne-Manneville and Hall, 2002).



Etienne-Manneville and Hall, *Nature*,2002.

Figure 1.9 - Rho-GTPases cycle
The GTP-bound Rho GTPase interacts with a wide spectrum of downstream effectors, initiating cascades that regulate events in the cytoplasm and the nucleus. The best characterised cytoplasmic function of Rho family proteins is their role as regulators of actin cytoskeletal organization. These dynamic Rho GTPase-controlled cytoskeletal rearrangement promote shape adjustment and regulate cell-cell and cell matrix contacts.

The Rho GTPases are important links between extracellular growth signalling pathways and the cytoskeleton, controlling both polymerization and branching of actin filaments and thereby cell locomotion, tumour growth, cell cycle progression, gene transcription and cell survival (Bishop and Hall, 2000; Hall, 1998; Ridley and Hall, 1992).

Rho GTPases are believed to stimulate plasma membrane protrusion by inducing actin filament nucleation and polymerization on or close to membranes. Their activity is regulated by signals originated from different classes of surface receptors including G-protein-coupled receptors, tyrosine kinase receptors, cytokine receptors and adhesion receptors. Important tools in the analysis of Rho protein functions are point mutated molecules making Rho constitutive active or dominant negative, and a number of bacterial toxins which covalently modifies the activity of the different Rho GTPases, either by

activation or inactivation. *Clostridium botulinum* and *Clostridium difficile* either activate, through deamidation, or inactivate, through glucosylation or ribosylation the different Rho GTPases. The exoenzyme C3 ADP-ribosyltransferase from *Clostridium botulinum* inactivates RhoA by ribosylation (Aktories, 1997; Aktories and Hall, 1989) and was used in my study.

1.7 Inflammatory Bowel Diseases (IBD)

IBD is a chronic relapsing inflammation of the gastrointestinal tract. IBD occurs in genetically predisposed individuals many of whom show loss of tolerance and mount an aberrant inflammatory response towards the normal bacterial flora of the gut (Monteleone et al., 2002a). The two main forms of IBD, Crohn's disease and Ulcerative colitis (UC), have many similarities, but there are also several clinical and pathological differences. Crohn's disease was first seen by German surgeon Wilhelm Fabry (1964) in 1623, and was later described by and named after the US physician Burril B Crohn (Crohn et al., 1984). Ulcerative colitis was first described by the British physician Sir Samuel Wilks in 1859 (S Wilks, *London Medical Times & Gazette* 2 (1859), p. 264).

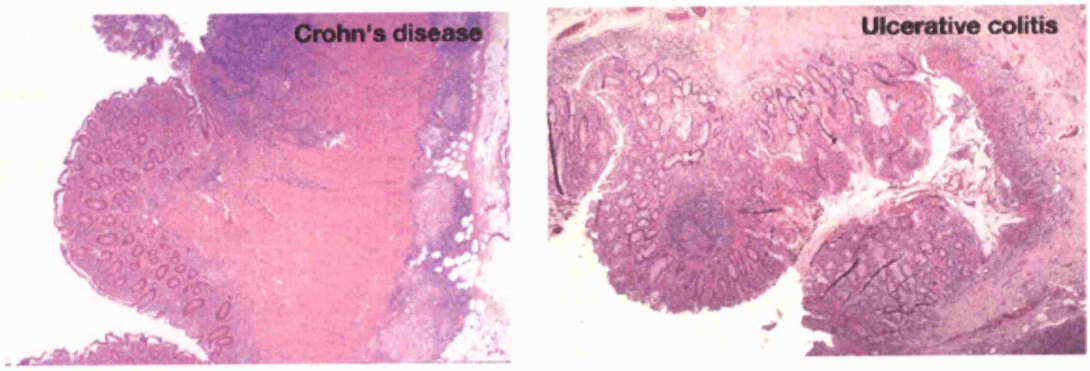
Crohn`s can affect any part of the intestine but most commonly affects the terminal ileum, cecum, perineum, and colon. The disease may be discontinuous. Crohn`s is typically a transmural (affecting all layers of the bowel wall) inflammatory process with lymphocytes, macrophages, granulomas together with fissuring ulcerations and strictures in some individuals

UC which can affect either the left or the entire colon usually involves rectum, extending proximally in a continuous manner. Usually it is restricted to the colon, and inflammation

is confined to the superficial (mucosal) layer of the intestine with infiltration of lymphocytes and granulocytes and loss of goblet cells with presence of ulcerations and crypt abscesses. Patients with UC suffer from chronic diarrhoea, and blood loss. In severe cases, this can lead to colonic perforation and can occur with transmural inflammation.

Patients with Crohn's present weight loss, abdominal pain and growth failure sometimes associated with narrowing of the gut lumen leading to strictures and bowel obstruction. Abscess formation, and fistulisation to skin and internal organs with fever and fatigue are observed. Extra-intestinal inflammatory manifestations in joint, eyes, skin, mouth and liver can occur in both forms of IBD. An increased risk of colon carcinoma is seen in longstanding IBD, in particular in UC (Bouma and Strober, 2003).

Both Crohn's disease and ulcerative colitis have a prevalence range of 10-200 cases per 100,000 individuals in North America and Europe. Disease incidence is the highest in developed, urbanized countries. The precise etiology of these diseases is unknown, but interplay of environmental risk factors and immunologic changes will trigger the onset of the disease in a genetically susceptible host (Vermeire and Rutgeerts, 2005).



Bouma G and Strober W, *Nature Review Immunology*, 2003

Figure 1.10 - Histological differences between Crohn's Disease and Ulcerative Colitis

Crohn's disease. It is characterised by transmural (affecting all layers of the bowel wall), dense infiltration of lymphocytes and macrophages; presence of granulomas; fissuring ulceration and submucosal fibrosis (see left-hand figure).
Ulcerative colitis. Inflammation affects superficial (mucosal) layers with infiltration of lymphocytes and granulocytes and loss of goblet cells. Presence of ulcerations and crypt abscesses (see right-hand figure).

1.7.1 Genetic factors in IBD

Epidemiological and family studies have provided overwhelming evidence that genetic factors have an important role in determining susceptibility to IBD (Bonen and Cho, 2003). The most compelling evidence comes from studies that are conducted with twins. The rate of concordance for Crohn's disease has been reported to be as high as 58% in identical twins, whereas the dizygotic-twin concordance is not significantly different from that for all siblings (Tysk et al., 1988). Reported concordance rates for monozygotic and dizygotic twins with ulcerative colitis range from 6–17% and 0–5%, respectively (Orholm et al., 2000; Tysk et al., 1988). Collectively, these observations strongly support the assumption that susceptibility to IBD, in particular Crohn's disease, is inherited. However, it also indicates that IBD is not inherited as a Mendelian trait, but rather has a complex

genetic basis with many contributing genes and a significant environmental influence. However the identification of genes involved in complex genetic disorders is quite complicated. A recent successful approach to identify genes involved in susceptibility to IBD has been to probe the genomes of families with many affected members using polymorphic micro satellite markers. These whole-genome screens are based on the principle that a marker that is located close to a disease gene is less likely to be separated during meiosis and will be co-inherited with the disease gene. Affected relatives (for example, affected siblings) will share alleles in excess of statistical expectation for markers close to a disease susceptibility gene(s). The analysis of large groups of affected siblings, therefore, allows the identification of broad genomic regions that contain a disease gene(s). Hugot et al (Hugot et al., 1996) used this approach to study families with patients with Crohn's disease. In this study a susceptibility locus was detected in the pericentromeric region of chromosome 16, now known as IBD1. Further analysis of this region identified a strong association with a single gene, NOD2, also known as caspase-recruitment domain protein 15 (CARD 15) (Hugot et al., 2001), which encodes an intracellular molecule of the NOD family that is thought to be involved in the recognition of bacteria (Girardin et al., 2003; Inohara et al., 2003). Sequencing of this gene in patients with Crohn's disease indicated a cytosine insertion that gives rise to a stop codon and a truncated NOD2 protein. These data indicated that NOD2 mutations occur exclusively in patients with Crohn's disease and not in patients with UC.

NOD2 is now recognised as a member of a large family of intracellular proteins, the NOD family, that are widely distributed in nature and includes the resistance proteins in plants that are involved in plant host defence against pathogens as already mentioned above.

Structurally, they consist of two amino-terminal effector domains, known as caspase-recruitment domains (CARDs), a central nucleotide-binding oligomerization domain (NOD) and multiple carboxyterminal leucine-rich repeat (LRR) domains that can function as an intracellular sensor of bacterial infection (Inohara et al., 2002; Inohara and Nunez, 2001).

NOD2 protein is expressed by monocytes, granulocytes, dendritic cells and, notably, also by epithelial cells (Gutierrez et al., 2002). *In vitro* studies have shown that stimulation of NOD2-transfected cells with bacterial proteins results in activation of the nuclear factor - κB (NF-κB) pathway. Such activation occurs through RICK (RIP-like interacting CLARP kinase receptor interacting protein 2), a serine/threonine kinase that phosphorylates inhibitor of NF-κB kinase (IKK) and, therefore, allows the transport of NF-κB to the nucleus (Gutierrez et al., 2002; Kobayashi et al., 2002). Initially, LPS was thought to be the bacterial component that is recognised by NOD2 protein, but in a recent work it has been shown that it is, in fact, muramyl dipeptide, a component of peptidoglycan that is associated with various bacteria and frequently contaminates preparation of LPS (Inohara et al., 2003). These components do not interact with TLR2, the extra cellular receptor for peptydoglican, and so NOD2 functions as an independent intracellular receptor. Interestingly, NOD2 has a high degree of polymorphism and is highly sensitive to the bacterial environment and undergoes frequent mutations to allow organisms in slightly different niches to cope with these environments, and that NOD2 has an important role in the natural immunity to potential bacterial invaders.

The findings that concern the activity of NOD2 and its dysfunction in Crohn's disease have led to several possible explanations as to the pathogenesis of Crohn's disease

associated with NOD2 mutations. The main hypothesis, at present, is that in the absence of NOD2 activity there is defective activation of macrophages owing to a marked NOD2-independent effector-T cell response. However, persistent intracellular infection of macrophages has not been detected in Crohn's disease, and other possibilities need to be considered. A second hypothesis is that in the absence of NOD2 expression by epithelial cells, microbial products that normally activate epithelial cells to secrete chemokines and defensins fail to do so, leading to first, the proliferation of bacteria in the crypts and second, loss of barrier function allowing marked stimulation of mucosal cells by mucosal antigens. A recent observation (Lala et al., 2003) that NOD2 is expressed by epithelial cells at the base of the villous crypts, known as Paneth cells, supports this possibility. A third hypothesis is that recognition of microbial peptides by NOD2 normally conditions APCs in a way that leads to their induction of regulatory and effector-T cell response, and so failure of this mechanism disrupts mucosal homeostasis.

Although the identification of NOD2 mutations has been an important scientific breakthrough, it should be emphasized that mutations in this gene occur in only a subgroup of patients with Crohn's disease. Indeed since the identification of IBD1 as the first susceptibility locus for Crohn's disease, several additional chromosomal loci have been identified, indicating the genetic complexity of IBD (Bonen and Cho, 2003).

1.7.2 Immunological response in IBD

An important advance in the study of IBD has been the discovery and subsequent analysis of a wide variety of mouse models of intestinal inflammation that resemble IBD (Blumberg et al., 1999; Strober et al., 2002). The differences in disease susceptibility and resistance among mouse strains used as model of mucosal inflammation offer the

opportunity to identify mouse genes that regulate the expression of genes with an important role in disease, and might therefore contribute to the identification of genes that are necessary for the occurrence of disease in humans. The current working hypothesis suggests that the disease is due to a dysfunctional interaction between bacterial microflora of the gut and the mucosal immune system resulting from a mucosal immune system that overreacts to normal constituents of the mucosal microflora, that is, in part, genetically determined.

In general, experimental colitis does not develop when mice are kept in a germ-free environment (Sartor, 1997). This is a demonstration that the normal mucosal microflora are required to initiate or maintain the inflammatory process, presumably by providing one or more antigens or co-stimulatory factors that drive the immune response in a genetically susceptible host. However, despite an extensive search, no specific pathogenic micro organisms have been associated with any of the models. In addition, although it is known that antigens from most resident bacteria do not take part in the disease process, there is little evidence that pathogenic antigens come from a single organism, or even a restricted group of organisms (Cong et al., 1998).

Despite the potential of antigens in the microflora to have pro- or anti-inflammatory effects, it is clear that these antigens introduced in the body at birth and have some degree of access to the internal milieu. As a result, they might be subject to the intra-thymic processing that allows the immune system to distinguish self from non-self antigens, and, if so, could be considered to be equivalent to self antigens (Karlsson et al., 1999). On the basis of this, it could be argued that by reacting to these antigens the organism is mounting

an autoimmune response and, by extension, the chronic mucosal inflammation of IBD could be thought of as an autoimmune disease.

In normal bowels, the immune reaction is sophisticatedly regulated while keeping a balance between the effectors and the regulators, and as a result, the homeostasis of the gut is maintained. In IBD this balance is broken and the inflammation observed occurs as a result of either excessive effector T cell function or deficient regulatory T cell function.

The former possibility is exemplified by models in which essential pro-inflammatory cytokines are overproduced. These include: mice in which TNF-α is overproduced due to a deletion (in the AU-rich regulatory elements (ARE) (TNF ARE mice); mice that are transgenic for signal transducer and activator of transcription 4 (STAT4), which have exaggerated IL-12 signalling that leads to increased production of interferon γ and TNF-α (Kontoyiannis et al., 1999; Wirtz et al., 1999).

The possibility that IBD is caused by defective T cell mediated regulation is indicated by the fact that most encounters with mucosal antigens do not induce effector cell responses, but instead induce negative regulatory T cell responses, anergy or deletion of antigen-specific T cells, a phenomenon known as "oral tolerance" (Strober et al., 1998). Such oral tolerance occurs in response to the mucosal microflora and establishes a mucosal homeostasis that ensures that most mucosal responses are self-limited and do not result in inflammation.

Failure of this mechanism can be a cause of mucosal inflammation and indeed, a surprising number of mouse models of IBD result from a defect in regulatory T cells. A notable example is mucosal inflammation that is induced in a severe combined immunodeficient (SCID) mouse by the adoptive transfer of naïve ($CD45RB^{hi}$) lacking

regulatory cells, which is prevented by the co-transfer of mature ($CD45RB^{low}$) cells that contain a regulatory T cell subpopulation (Powrie et al., 1993; Powrie et al., 1994). In addition, intestinal inflammation occurs in models in which there is a clear deficiency in the production of a known regulatory cytokine, such as IL-10 knockout mice (Kuhn et al., 1993) or mice with defective transforming-growth factor-β (TGF-β) signalling (Gorelik and Flavell, 2000). Finally, colitis occurs in mice in which regulatory T cells fail to develop properly. For example, a line of CD3ε transgenic mice have an aberrant thymic microenvironment and a population of T cells develop and can induce severe colitis, this can be prevented by regulatory T cells that have undergone normal thymic development (Hollander et al., 1995). In addition to preventing nascent inflammation, it has recently been shown that regulatory T cells can reverse established inflammation. Administration of $CD25^{+}$ regulatory T cells to SCID or recombinase-activating gene 1 (Rag1)-deficient mice cures colitis that has been previously induced by the administration of $CD45RB^{hi}$ T cells.

Recent work has established that mucosal epithelial cells can recognize microorganisms and/or substances that are produced by microorganisms in the mucosal lumen, at least in part, through their expression of TLRs (Cario et al., 2000). Under appropriate conditions, epithelial cells can react to these microorganisms with the production of cytokines, chemokines and other pro- and anti-inflammatory substances (Gewirtz et al., 2001; Maaser and Kagnoff, 2002). It is probable that this function of epithelial cells has an important role in mucosal host defence, and that they are also involved in the initiation and/or potentiation of mucosal inflammation and IBD (Cario and Podolsky, 2000). It is clear that these active functions of epithelial cells are crucial for the maintenance of their

essential passive function — that is, the ability to function as a barrier to luminal micro organisms that could otherwise stimulate chronic inflammation (Podolsky, 1999).

Although mouse models have led to important insights into the pathogenesis of mucosal inflammation and the mechanisms that might underlie IBD, to gain an unequivocal understanding of the disease, one must look at the characteristics of patients suffering from IBD.

Regardless of the particular defects, the disease process is inevitably channelled into a final common immunopathologic pathway, comprised of either a Th1 type T cell mediated inflammation (in Crohn's disease) (Bouma and Strober, 2003; Shanahan, 2000) with large production of IFN-γ, TNF-α and IL-12, which is a critical Th1 polarizing factor (Monteleone et al., 1997); or a Th2 type T cell mediated inflammation (UC) with production of IL-4 and particularly IL-5 (Heller et al., 2002).

The data that support that Crohn's disease is a Th1 mediated inflammatory disease are quite substantial. Various immunohistological studies indicate that *in situ* IL-12 is overproduced by macrophages in Crohn's disease (Monteleone et al., 1997), but not in UC, and macrophages that are isolated from the inflammatory lesions of patients with Crohn's disease produce increased amounts of IL-12 *ex vivo* (Parronchi et al., 1997), whereas macrophages that are isolated from patients with UC produce decreased amounts of IL-12, compared with those from normal tissues. In addition, nuclear extracts of T cells from the affected tissues of patients with Crohn's disease contain increased amounts of activated STAT4 and the transcription factor T-bet, which is indicative of IL-12 signalling, and T cells isolated from these tissues express increased amounts of the IL-12Rβ2 chain, a characteristic of Th1 cells (Neurath et al., 2002; Parrello et al., 2000). In

view of these findings, it is not surprising that T cells isolated from the affected tissues of patients with Crohn's disease or clones that are derived from such cells produce markedly increased amounts of IFN-γ together with markedly decreased amounts of IL-4, compared with controls (Fuss et al., 1996). This circumstantial evidence in support of the causal role of Th1 mediated processes in Crohn's disease is perhaps supported by the finding that patients treated with an antibody specific for the p40 chain of IL-12 leads to a marked amelioration of inflammation in most patients. The clinical improvement is associated with a reduction in both the production of IL-12 and IFN-γ by mononuclear cells that are isolated from the affected tissues.

Whereas there is abundant evidence that T cells from lesions in Crohn's disease produce increased amounts of IFN-γ, the signature Th1 cytokine, there is no evidence that T cells from UC produce increased amounts of IL-4, the definitive Th2 cytokine. Despite this difficulty, there is, in fact, considerable evidence that ulcerative colitis is a Th2 mediated disease. First, UC, to a greater extend than Crohn's disease, is associated with the production of various auto antibodies, such as anti-neutrophil cytoplasmic antibody (pANCA) and anti-tropomyosin (Das et al., 1993; Saxon et al., 1990).

As Th2 cells provide more efficient responses than Th1 cells do, the presence of auto-antibodies might be indicative of a Th2 rather than Th1 mediated immune response. Second, studies on the production of immunoglobulin subclasses show that subclasses that are associated with Th1 cell response, such as IgG2 antibodies, are increased in Crohn's disease and Th2 related subclasses, such as IgG1 and IgG4 antibodies, predominate in UC (Kett et al., 1987). Third, although UC has not been associated with increased secretion of

IL-4, it has been associated with increased secretion of IL-5, another Th2 cytokine (Fuss et al., 1996).

1.7.3 Immunotherapy in IBD

Identifying the particular antigen(s) that drive the Th1 inflammatory response in the face of the myriad of potential antigens in the gut has proven difficult. Nevertheless, the likelihood is that bacterial antigens are involved, because stimulation of mucosal CD4 cells from Crohn's disease patients with extracts of their own commensal flora can induce interferon-γ production (Duchmann et al., 1995), and in murine colitis, flagellin from commensal bacteria also activates mesenteric lymph node CD4 cells (Lodes et al., 2004). Clinical observations also support a role for antigens derived from the commensal flora.

In human IBD tissue, $CD4^+$ T cells represent the vast majority of activated mononuclear cells infiltrating the gut. A large proportion of T cells bear the phenotypic characteristics of circulating naïve lymphocytes, and they are recruited from the blood into the intestinal mucosa, probably as a result of an enhanced expression of adhesion molecules and chemokines in the inflamed gut of IBD patients (Fiocchi, 1998).

Despite redundancy among mediators of inflammation, a hierarchy of importance has emerged with TNF-α as a key effector and regulatory molecule in Th1 responses in Crohn's disease (Shanahan, 2000).

Whereas increases in the levels of the putative initiating cytokines, such as IFN-γ, are relatively modest (~3 fold), increases in the levels of the downstream pro-inflammatory cytokines are more marked (~10-20 fold). This argues for the occurrence of an as yet poorly understood multiplier effect in IBD inflammation, in which even small increases in initiating cytokines give rise to large increases in downstream cytokines.

Although the predominant immune response in UC is Th2 skewed, it has been shown that TNF-α may also play a role in its pathogenesis (Murch et al., 1991; Murch et al., 1993). Blockade of TNF-α signalling with anti TNF-α antibodies (Infliximab) has already proven to be a highly effective treatment in Crohn`s disease. Such treatment achieves positive effects in 60% of patients; however, the exact mechanism is unclear (Atreya et al., 2000; Van den Brande et al., 2003) and its efficacy is likely attributable to more than simple blockade of the TNF-α signalling.

It has been shown that in UC Infliximab is more effective than placebo for the treatment of moderate-to-severe disease, achieving clinical remission in 40% of the patients at approximately 9 months of follow-up (Gisbert et al., 2007).

Infliximab is a chimeric IgG1 monoclonal antibody against TNF-α, which was created in late 1980s. Infliximab is a mouse/human chimeric monoclonal antibody of which 25% is mouse peptide sequence. The murine component is ascribed to its immunogenecity, such as infusion-related reactions and serum sickness-like diseases. In such immunological reactions, the formation of antibodies against Infliximab, called human anti-chimeric antibodies (HACA) is of particular concern as the presence of HACA is associated with an increased frequency of infusion reactions and the reduction in efficacy (Baert et al., 2003). Most of the other anti-TNF reagents are modified by a reduction of the mouse peptide sequence or are completely humanized in order to reduce the immunogenicity. Not all of the other anti-TNF reagents have been proven to be as effective in the treatment of Crohn's as Infliximab, and the efficacy of such reagents seems to be dependent, not only on the ability to neutralize soluble TNF-α, but also on the capacity to bind to the

membrane-bound TNF-α on the cell surface, thereby mediating the apoptosis of the effector cells (Van den Brande et al., 2003).

Previous studies in autoimmune diseases like Rheumatoid arthritis (RA) showed that TNF-α inhibits the suppressive function of $CD4^+CD25^+$ T Regulatory cells and down-regulates the master gene FOXP3 specific of these cells (Valencia et al., 2006).

In recent work it was observed that this defect can be reversed after Infliximab treatment (Nadkarni et al., 2007). These data demonstrate that Treg cells can be induced, and tolerance restored, by targeting specific pro-inflammatory cytokines such as TNF-α.

In UC currently the most widely used aminosalycilic acid derivatives, steroids, an anti-proliferative agent (azathioprine) and immunosuppressive regimes including a calcineurin inhibitor (cyclosporine). Recently other therapies have been explored, with particular targeting of the pro-inflammatory cytokine IL-2 receptor (CD25). IL-2 is secreted by activated T lymphocytes and acts via the high-affinity IL-2R to promote cell survival and proliferation.

In UC, inflammation appears to be driven by activated T lymphocytes. The role of T cells as mediators of inflammation and tissue injury in UC is supported by the fact that cyclosporine A is effective in the treatment of severe disease. In addition, in both Crohn`s and UC, $CD25^+$ cells are present as aggregates in the lamina propria and constitute a large proportion of lamina propria mononuclear cells (Choy et al., 1990).

Current medical treatment options for UC progress from aminosalicylates for mild disease to systemic steroids for moderate disease to cyclosporine for severe disease. An anti-CD25 monoclonal antibody (Daclizumab), has been investigated as a potential treatment for UC with mixed results (Van et al., 2003; Van et al., 2006). Neither study evaluated steroid

refractory UC subjects nor, in addition, while daclizumab saturated circulating CD25, it did not appear to saturate CD25 cells in rectal tissue biopsies, suggesting that daclizumab did not reach the target tissue in sufficient concentrations.

Recent studies have revealed a functional role for CD25 expression on $CD4^+$ T reg cells such that interruption of the IL-2/IL-2R signaling pathway blocks Treg effector function potentially via alterations in the expression of the glucocorticoid-induced TNFR-family gene (GITR) (Kohm et al., 2005; Thornton et al., 2004). Accordingly, a number of groups have targeted CD25 as a mechanism of depleting Treg cell and studying resultant effects on T cell activation, trafficking, and/or effector function. It is widely believed that injection of anti-CD25 mAb result in the rapid and efficient depletion of $CD4^+CD25^+$ Treg cells as determined by secondary immunostaining with mAb anti CD25 (Onizuka et al., 1999; Shevach, 2002). In recent studies it has been reported that *in vivo* injection of anti CD25 mAb in mice resulted in the functional inactivation but failed to physically deplete $CD4^+CD25^+$ Treg cell resulting in an exacerbation of acute clinical experimental autoimmune encephalomyelitis (EAE) (Kohm et al., 2006). Supporting this, mice receiving anti-CD25 mAb treatment displayed significantly lower numbers of $CD4^+CD25^+$ T cells but no change in the number of $CD4^+Foxp3^+$ Treg cells. Other studies in mice also revealed that transient depletion of $CD4^+CD25^+$ Treg in vivo using anti CD25 resulted in severe spontaneous autoimmune thyroiditis (SAT) (Yu et al., 2006).

The same was expected in UC but recent clinical studies by our group have shown that the use of anti-CD25 antibody Basiliximab was efficacious in the improvement of fulminating ulcerative colitis (UC) (Schwarzer et al., 2006).

Basiliximab (Simulect) is a chimeric (murine/human) monoclonal antibody ($IgG_{1\kappa}$) that is product by recombinant DNA technology. It has been demonstrated to be safe and well tolerated in clinical studies after it has been previously used as immunosuppressive drug to prevent allograft rejection in almost all human transplant recipients. Specifically, Basiliximab binds to and blocks the IL-2 receptor α-chain (IL-2R/CD25) expressed on the surface of activated and regulatory T cells and it therefore competes with IL-2 in binding to the high-affinity receptor (Amlot et al., 1995).

1.8 Aims of the study

To analyze the mechanism of innate immune response activation in Celiac Disease (CD).

- Understanding the mechanisms leading to epithelial changes in CD and setting of adaptive responses by looking at the role of Rho-a involvement in gliadin induced actin reorganization, tyrosine phosphorylation and apoptosis in CD.

To explore the cross talking between innate immune system and acquired immune regulatory networks

- Investigation of the role of FOXP3+ Regulatory T cells in IBD and CD.

To exploit the potential of regulatory networks in immunotherapy for IBD

- Investigation of the role of FOXP3+ Regulatory T cells after anti-TNF-α therapy in Crohn's Disease.
- Investigation of the modulation of FOXP3+ Regulatory T cells after anti- IL2R (CD25) therapy in Ulcerative Colitis.

Chapter 2 – Materials and Methods

2.1 Reagents and solutions

Dulbecco`s modified Eagle`s medium, Ham`s F12 medium (DMEM/F12), penicillin-streptomycin (Pen/Strep), heat-inactivated Fetal Calf Serum (HI-FCS), trypsin/ethylenediaminetetraacetic acid (EDTA) (1x), Phosphate buffer saline (PBS), NCTC-135, Fetal Bovine serum (FBS) and L-Glutamine were all purchased from Invitrogen (Paisley, UK).

Trowell` T8 medium was purchased from Eurobio (Courtaboeuf Cedex B, France).

Paraformaldehyde (PFA), Triton X100, Bovine Serum Albumin (BSA), Dimethyl sulfoxide (DMSO), Meyer`s Hematoxylin, agarose, ethidium bromide, Tris Borate EDTA (TBE), Magnesium Chloride, Phalloidin Fluorescein Isothiocyanate Labeled (FITC), Phalloidin Tetramethylrhodamine B isothiocyanate (TRITC), Staphylococcal Enterotoxin B (SEB) were all purchased from SIGMA (Poole, Dorset, UK).

The optimal cutting temperature (OCT) compound was purchased from Tissue Tek; Miles Laboratories, Elkhart, IN),

Cytochalasin D and Y27632 (ROCK-I inhibitor) were purchased from Calbiochem (Darmstadt, Germany).

Exoenzyme C3 transferase was purchased from Cytoskeleton, Universal biological (Cambridge, UK).

The vector pRK5-myc, encoding the recombinant C3-transferase from *Clostridium botulinum*, a Rho-selective inhibitor was a kind gift from Dr Alan Hall, Medical Research Council, University College London, UK.

Gliadin was extracted from pure hexaploid bread wheat (Triticum aestivum variety San Pastore), submitted to peptic-tryptic digestion and was akind gift from Luigi Maiuri, University of Naples, Italy.

Gliadin peptide p31-43 was synthesised by the Advanced Biotechnology Centre (Imperial College London, UK).

Normal Goat serum, peroxidase blocking solution, StreptavidinFITC and Streptavidin HRP were purchased from DAKO Cytomation, Ely, Cambridgeshire, UK).

Annexin V FITC and Propidium Iodide were purchased from R&D systems, Abington, UK).

12 mm Transwell, Polyester membrane (Costar 3460) were purchased from Corning, Schiphol- Rijk, The Netherlands)

Millipore millicell-ERS voltohmeter was purchased from Millipore (Bedford, MA).

2.2 Antibodies

Table 2.1 Antibodies for flow cytometry, immunocytochemistry, immunohistochemistry

Name	Conjugate/Isotype	Sources
Anti myc Tag (clone: 9E10)	mouse IgG1	Dr Alan Hall (MRC, UCL, London)
Anti- phosphotyrosyne (clone: 4G10)	mouse IgG2bκ	Upstate (Lake placid, NY, USA)
Anti phosphor FAK (Tyr397)	Rabbit antiserum	Upstate (Lake placid, NY, USA)
Anti FOXP3 (clone: 236A/E7)	mouse IgG1	Abcam (Cambridge, UK)

Rabbit anti mouse	FITC	DAKO (Ely, UK)
Goat anti Rabbit	Biotin	DAKO (Ely, UK)
Goat anti-mouse	Biotin	DAKO (Ely, UK)
Streptavidin	FITC	DAKO (Ely, UK)
Streptavidin	HRP	DAKO (Ely, UK)

2.3 Cell Culture and culture conditions

The human colonic adenocarcinoma-derived T84 epithelial cell line were maintained in a 1:1 mixture of DMEM/F12 medium with 10% Pen/Strep, supplemented with 10% HI-FCS and kept in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

For microinjection and staining experiments, cells were seeded at the density of 2 $X10^4$ cells/ml on glass slides in round wells.

For apoptosis experiments, to form a monolayer, cells were suspended with trypsin/EDTA, and seeded on Transwells, at the density of $2,5X10^5$ cell/well until became confluent.

For apoptosis study T84 cells were cultured as a polarized monolayer and exposed to the gliadin peptide 31-43 for 24h. Monolayers were used 3-4 days after plating, the medium of the upper and lower filter chambers was changed every 24h and the formation of a stable monolayer was assessed by monitoring the Transepithelial resistance (TER) that was measured using a Millipore millicell-ERS voltohmeter. Experiments were performed when TER was $2000\Omega/cm^2$.

The gliadin peptide 31-43 was used at the concentration of 20µg/ml either for apoptosis evaluation or for actin cytoskeleton studies. This peptide concentration was the same used for *ex-vivo* culture of CD biopsies samples in other previous studies (Maiuri et al., 2003;

Maiuri et al., 2005). For peptide stimulation, other cells such as the mouse fibroblast NIH-3T3 were used as control. In these cells no effect either on apoptosis or cytoskeleton modifications was observed. As negative control for the specific peptide p31-43 effect on the epithelial T84 cells, the immunodominant peptides pα2 and pα9 were used.

To determine the optimal timings where either the effect on cytoskeleton or apoptosis were more visible, a time course of the peptide p31-43 was performed.

For the studies on the cytoskeleton the time course of peptide stimulation was: 5 min, 10 min, 15 min, 20 min, 30 min, 40 min and 1 h. The optimum was at 20 min and it was used for the subsequent experiments.

For the visualization of apoptosis the time course of peptide stimulation was: 12h, 24h and 48 h. The optimum was at 24h and it was used for the subsequent experiments.

The effect of cytoskeletal disruption was evaluated pre-treating the cells with a fungal metabolite, an actin polymerization inhibitor, Cytochalasin D, prior the gliadin peptide 31-43 treatment. To determine the optimum inhibition effect, different concentrations were used starting from 100μM, 50μM, 20μM, 10μM and 1μM. The optimum concentration of CytochalasinD was found at 20 μM.

T84 cells then were pretreated with the Rho-A inhibitor, exoenzyme C3 transferase. In this case also different concentrations were used to decide the optimum condition starting from 50μg/ml, 10μg/ml, 5 μg/ml and 1μg/ml. The optimum concentration of Rho-A inhibition was found at 5 μg/ml.

For the ROCK-I inhibitor, Y-27632, I have done the same starting from 100μM, then 50μM, 20μM, 10μM and 1μM. The optimum inhibition was found at 10μM.

A time course also was performed to decide the optimum time of inhibition, pre-treating T84 cells for 24h, 12h, 1h, 30min and 15 min, prior the peptide p31-43 stimulation. The optimum time was found at 30 min.

The gliadin peptide 31-43 was added at the concentration of 20 μg/ml either to the apical or to the basolateral well. Untreated cells were considered as internal control.

2.4 Microinjections

T84 cells were plated at 5 X 10^4 cells per 35-mm culture dish on round coverslips.

Microinjections were performed as described by Hall and Ridley (Ridley and Hall, 1992) using an Eppendorf microinjector 5442 coupled to an Eppendorf Micromanipulator 5170 attached to an Olympus inverted microscope.

About 50 cells per well were microinjected into the nucleus within a 20 min period with the vector pRK5-myc at 200 μg/ml, encoding the recombinant C3-transferase from *Clostridium botulinum*, a Rho-selective inhibitor.

The cDNA encoding the C3 transferase in pRK5-cMyc (bacterial expression system), was a kind gift from Dr Alan Hall, Medical Research Council, University College London, UK and was purified as described (Dillon ST, Feig LA, Methods Enzymol, 256:174-184, 1995).

As control, a vector encoding EGFP, at 200μg/ml, was microinjected in the nuclei of the cells.For each dish, non-injected cells were used as internal control.

Microinjected cells were incubated at 37°C for 4h before the treatment with the peptide p31-43 (20μg/ml) for 20 min.

2.5 Immunofluorescence

To visualize the distribution of the filamentous (F) actin, after Cytochalasin D treatment, microinjection with the vector pRk5mycC3 or with EGFP and stimulation with the peptide 31-43, T84 cells were fixed in 4% PFA/PBS for 10 min at room temperature and permeabilised for 10 min with 0,2% Triton X-100. Cells were incubated in 1% BSA/PBS for 30 min to block nonspecific bindings and then incubated with anti Myc tag mouse antibody (clone 9E10) for 1h at room temperature to distinguish the cells that had been microinjected.

Cells were incubated afterwards with 0,3 μM Phalloidin TRITC plus the secondary antibody Rabbit anti mouse FITC conjugated for 30 min at room temperature.

To quantify F-actin levels, cells were fixed, permeabilized, blocked as above and incubated with FITC-Phalloidin 0.3 μM for 30 min at room temperature.

For the evaluation of the phosphorylation level, cells were incubated with a mAb anti phosphotyrosine 4G10. To determine the optimism mAb concentration to use, titrations were performed. T84 cells were incubated with 50 μl mAb at following dilutions 1:20, 1:50, 1:80, 1:100, 1:200, 1:500. The maximum expression of saturation of mAb binding was quantitated by confocal microscopy. The optimum was at 1:80. The cells were incubated for 1 h at room temperature followed by incubation with a secondary antibody rabbit anti mouse FITC.

For the evaluation of Focal Adhesion Kinase phosphorylation cells were incubated with a rabbit polyclonal antibody anti phosphor FAK (Tyr^{397}). Titration of the antibody was performed in this case as well as for the previous antibody and the optimum was at 1:200. The cells wre incubated for 1h at room temperature followed by incubation with a

secondary antibody Goat anti Rabbit Biotinylated, followed by Strepdavidin FITC and Propidium Iodide for nuclei counterstaining.

Immunofluorescence was visualized with a Confocal microscope (Leica TCS SP2), magnification 200X.

2.6 Annexin V staining

For apoptosis evaluation T84 cells were collected and resuspended in 1x binding buffer [0.01 M HEPES/NaOH (pH 7.4), 0.14 mM NaCl, and 2.5 mM $CaCl_2$] at a concentration of 1 x 10^6 cells/ml. Subsequently, 100 µl of the cell suspension was transferred to a 5-ml tube and Annexin V (5 µl) and PI (10 µl) were added according to manufacturer's instruction.. The cells were incubated at room temperature for 15 min, after which 400 µl of 1x binding buffer was added, and apoptosis, as judged by Annexin V staining, was analyzed by flow cytometry.

Two-colour cytometric analysis (Fluorescence-Activated Cell Sorting [FACS]) was performed on a Beckman Coulter with an Argon laser tuned at 488 nm. The data were analysed using WinMDI software.

2.7 Patients and biopsy specimens

Biopsy specimens from patients with IBD, celiac disease and controls were taken during endoscopy at the Gastroenterology Unit, Great Ormond street Hospital, London.

Fully informed consent was obtained from the parents of all patients. Ethical approval was granted by the Great Ormond Street Hospital REC.

Celiac disease, Crohn`s disease and Ulcerative colitis were diagnosed by established clinical and histopathological criteria.

Duodenal specimens from 10 patients with untreated celiac disease and with positive autoantibodies anti tissue transglutaminase (mean age 9.8 years, range 6-15) and from 6 controls (children being investigated for oesophagitis) (mean age 11.25 years, range 6-17) were obtained at the duodenal-jejunal flexure by peroral biopsy. The CD patients after the diagnosis were put in Gluten free diet.

Colon specimens from 7 Crohn`s patients treated with Infliximab (mean age 13.4 years, range 11-16), from 5 Crohn`s patients treated with conventional therapies that involve remission induction with enteral feeds followed by remission mainteinance with azathioprine and a 5-aminosalicylate preparation (mean age 13.5 years, range 10-16) and from 4 controls (children being investigated for constipation in whom inflammation was absent in routine laboratory histology) (mean age 10 years, range 8-11) were taken by colonoscopy.

Colon biopsies also from 4 patients with Ulcerative Colitis (mean age 13.5 years, range 12-16) were obtained by colonoscopy. These children were diagnosed with acute severe UC and were at high risk of colonic perforation. At the beginning they were treated with steroids but none of them responded satisfactorily to intravenous steroids alone. Subsequently they received intravenous ciclosporin. All four patients demonstrated, using the clinical scoring system, an element of response to calcineurin inhibition although further intervention was indicated.

All specimens were collected in ice-chilled tissue culture medium and cultured within 20 minutes. Each biopsy specimen was washed in 0.15 mol/L sodium chloride and examined

with a dissecting microscope. One specimen from each patient was oriented and embedded in OCT, snap frozen in isopentane cooled in liquid nitrogen, and then stored in liquid nitrogen until cryosectioning.

For RNA extraction one specimen also was snap frozen in liquid nitrogen.

2.8 Mucosal Tissue Culture

Mucosal samples from each patient were cultured in tissue medium containing 65% Trowell medium, 20% NCTC, 15% FBS, Penicillin 2000 U and Streptomycin 2 mg, 1 mM Glutamine and placed on a stainless steel mesh positioned over the central well of an organ culture dish with the mucosal surface of the biopsy specimens uppermost.

Biopsies from celiac patients were cultured in medium alone or with the addition of Peptic-tryptic (PT) digest of gliadin at 250µg/ml for 24h as previously performed in other *ex-vivo* studies (Maiuri et al., 2003).

Samples were also cultured with the addition of Staphylococcal Enterotoxin B at 200pg/ml for 24h.

To determine the optimum SEB concentration to use, titrations were performed. Biopsy samples were cultured with SEB for 24h with the following concentrations: 1µg/ml, 500 ng/ml, 100 ng/ml, 1ng/ml, 500pg/ml, 200 pg/ ml, 100 pg/ml.

2.9 Immunostaining on mucosal samples.

Five µm-thick cryostat sections of each intestinal mucosa sample from the duodenum of celiac patients and controls and from the colon of Crohn's patients and controls were cut at the cryostat and placed on poli-Lys coated slides and allow drying at room temperature.

Slides were fixed in 4% PFA for 10 minutes then washed in Tris-buffered saline (TBS) (pH 7.4). Sections were blocked for non specific binding with 10% goat serum for 20 min at room temperature. They were then incubated overnight at +4°C with a mouse monoclonal antibody anti Foxp3 (clone 236A/E7) followed by incubation with a secondary antibody Goat anti Mouse Biotynilated followed by Strepdavidin FITC.

Paraffin embedded sections obtained from UC patients were de-waxed in hystoclear and then re-hydrated in a decrescent alcohol series (100%, 95%, 85% and 70%) until the last wash in water. Antigen retrieval was done in 0.01M citric acid buffer pH6 in microwave oven.

Sections were blocked for non specific binding with 10% goat serum for 20 min at room temperature. They were then incubated overnight at +4°C with a mouse monoclonal antibody anti Foxp3 (clone 236A/E7) followed by incubation with a secondary antibody Goat anti Mouse Biotynilated followed by Strepdavidin FITC.

Immunofluorescence was visualized with a Zeiss Axioplan2 imaging microscope.

For a quantitative analysis, sections were stained by immunohistochemistry as already mentioned above but they were pre-treated with Peroxidase blocking solution. As final step to detect the positive staining, the secondary antibody biotinylated was revealed using Streptavidin HRP and nuclei were counterstained with Meyer`s Hematoxylin.

The counts of the numbers of FOXP3 positively stained cells were counted under a light microscope through a calibrated ocular graticule fitted into one of the eye pieces of the microscope and aligned parallel to the muscularis mucosa.

These counts allowed the analysis of the tissue with reference to high power fields (HPF). A cell was regarded as exhibiting positive staining if a nucleus was identified in association

with appropriate staining. Scoring the sections was achieved by counting the number of positively staining cells per HPF. Each HPF measured 0.25 mm^2, giving a conversion factor of 0.0625, which was used to express the result in mm^2. In the specimen, the positive cells in the lamina propria, comprising at least 10 fields, were counted and expressed as the mean number of positive staining cells/mm^2 in the lamina propria.

2.10 RNA isolation from duodenal biopsy samples

The samples were taken immediately from the gastroscopic forceps with pre-cooled sterile injection needle and snap frozen in liquid nitrogen. The frozen sample was cut with a sterile blade into the smallest visible pieces and dropped into cool Trizol Reagent (Gibco).

RNA was isolated as described in protocol of Gibco Trizol Reagent.

The quality of the RNA is checked by PCR using house-keeping gene (GAPDH).

2.10.1 RNA and cDNA synthesis

Total RNA was extracted using an RNeasy Mini Kit (Quiagen, Germany) according to the manufacturer's instruction. The amount and purity of the obtained RNA was determined by measurement of optical density at 260 and 280 nm.

10 µl total RNA was used for first/strand cDNA synthesis with oligo/dT.

2.10.2 Reverse Transcriptase Polymerase chain reaction (RT-PCR)

Primers sequences for Foxp3 were:

Foxp3 Forward: 5`-TCA AGC ACT GCC AGG CG- 3`

Foxp3 Reverse: 5`- CAG GAG CCC TTG TCG GAT-3`

The housekeeping gene GADPH was used as a positive control for the PCR.

GAPDH primers sequences were:

Forward: 5`- AGC CAC ATC GCT CAG ACA C -3`

Reverse: 5`- GAG GCA TTG CTG ATG ATC TTG -3`

A total reaction volume of 50 µl consisting of the following reagents was used:

cDNA	2 µl
$MgCl_2$	1.5 mM
dNTPs	0.1mM
DMSO	5%
NH_4 Buffer 10X	1.5µl
Oligo For	1 µM
Oligo Rev	1 µM
TaqPol	0.1µl

DNase/RNase-free water was used to top up the reaction volume to 50µl.

The PCR conditions were as follow:

92C	2'30''
92C	30"
55C	30"
72C	30"
Repeated 40 times	
72C	5'
25C	5''

Gel electrophoresis of 10µl PCR product alongside a 100kb ladder was performed on 2% agarose Tris-Borate EDTA (TBE) (1M Tris-EDTA, 0.9M Boric acid, 20 mM EDTA pH8, 10 X solutions) gel at 100 V for 1h. The gel was stained in 0, 5 µg/ml Ethidium bromide in TBE and visualized by ultraviolet illumination and image capture using Biorad Chemidoc.

2.11 Statistical Analysis

Parametric data are expressed as the mean ± SD of data obtained from three to four separate experiments. The statistical significance of differences between group means was determined using Student's *t* test using Prism 4 software (Graph Pad, San Diego, California, USA). A *P*-value of <0.05 was considered statistically significant differences between groups.

Chapter 3 – Rho involvement in gliadin induced innate immune response in epithelial cells of CD mucosa

3.1 Introduction

The intestinal absorptive cells (enterocytes) are in contact at their basal pole with a well developed extracellular matrix synthesized both by epithelial cells and the underlying mesenchymal cells. This exoskeleton interacts with cell surface receptors such as integrins, which transduce information from the extracellular environment to the intracellular compartment. These interactions play a key role in the maturation, migration, and renewal of the intestinal epithelium. The apical pole of the enterocytes forming the intestinal epithelium is in continuous contact with the intestinal luminal content.

The intestinal epithelium represents the first line of defence with the activation of the innate immune response to food antigens and bacteria.

One of the early events observed in CD, after gluten challenge, is the activation of the innate immune response by the epithelial barrier. These events precede the crucial activation of pathogenic $CD4^+$ T cells and are induced by fragments of gliadin not recognized by the adaptive immune system.

The early changes observed in the enterocytes of the intestinal mucosa of celiac patients, are represented by the reorganization of the actin filaments, with induction of stress fibers (Bailey et al., 1989; Clemente et al., 2000; Holmgren et al., 1995).

Regulation of most actin-dependent processes has been demonstrated to be mediated by Rho family GTPases in many different cell types, including epithelial cells (Hall, 1998).

Rho-A GTPases in particular control actin reorganization with induction of stress fibers (Hall, 1998).

The RhoA protein was first suggested to be involved in regulating actin polymerization based on studies with the selective inhibitor C3 ADP ribosyl-transferase isolated from *Clostridium botulinum* (Aktories and Hall, 1989).

C3 ADP ribosyl transferase binds to NAD^+ and catalyses the addition of an ADP-ribose onto an asparagine residue at position 41 of Rho (Narumiya et al., 1988). It appears that ADP-ribosylation of Rho at this residue renders the GTP-binding protein biologically inactive (Paterson et al., 1990). This was concluded from the finding that activated Rho protein, microinjected after in vitro ADP-ribosylation, loses its ability to induce formation of stress fibers (Paterson et al., 1990). Because Asn-41 is located in the so-called effector region of Ras-related GTP-binding proteins, it has been suggested that ADP-ribosylation disturbs the interaction with a putative effector.

This activity of C3 has been exploited to reveal that Rho proteins function to modulate the polymerization of actin in cells (Hall, 1992). In particular, when Rho is inactivated in a cell by C3-induced ADP-ribosylation, actin filaments depolymerise (Paterson et al., 1990).

Clemente et al. have recently used the rat intestinal IEC-6 cell line to investigate the effect of gliadin on the rearrangement of the actin cytoskeleton (Clemente et al., 2003).

Work from this laboratory (Maiuri et al., 2005) has already demonstrated that the activation of this early immune response is due to a particular gluten peptide that is not recognised by the $CD4^+$ T cells of the lamina propria. These early modifications were also controlled by antibodies able to control another type of tissue transglutaminase (tTG) that is not the autoantigen or the enzyme able to catalyze the modifications of gluten peptides so that they become immunodominants and are recognised in the HLA-DQ2/DQ8 context in CD.

This other type of tTG is present on the surface of the intestinal epithelium and is called surface tissue transglutaminase (stTG).

Previous studies have demonstrated that by controlling stTG it is possible to control epithelial modifications such as actin reorganization, protein tyrosine phosphorylation and apoptosis (Maiuri et al., 2005).

It has recently been observed that cell surface tTG can activate Rho-A (Janiak et al., 2006) either via integrin clustering or by enzymatic transamidation (Singh et al., 2001; Singh et al., 2003).

The work described in this chapter seeks to determine which cellular pathway induces the control of the innate immune response induced by gluten peptide p31-43 and whether the modifications induced by stTG, such as actin reorganization, tyrosine phosphorylation and apoptosis, observed in the epithelium of CD mucosa is regulated by Rho-GTPases. The investigations shed further light on the molecular mechanisms of the innate immune system in CD.

3.2 Results

3.2.1 Cytoskeletal modification in intestinal epithelial cells

Several groups have reported that the innate immune response to gliadin peptides (seq 31-43 or 31-49) is characterized by specific changes such as induction of actin reorganization with induction of stress fibers (Bailey et al., 1989; Clemente et al., 2003; Clemente et al., 2000; Holmgren et al., 1995).

Actin stress fibers consist of transient, contractile, long bundles of filaments that traverse the cell and are anchored to the plasma membrane through multimolecular protein complexes called focal adhesions; at these sites the cell contacts the substratum through transmembrane integrins (Burridge et al., 1987), which transduce information from the cell environment to the intracellular compartment. Contraction of anchored stress fibers allows the cell to exert tension on the substratum, a process that is important in wound healing and morphogenesis (Horwitz and Thiery, 1994).

For these studies I took advantage of the responsiveness to gluten peptide p31-43 of T84 colonic epithelial cell lines. The T84 epithelial cell line is derived from a colonic carcinoma and reproduces the crypt cell phenotype in many respects. Morphologically, T84 cells retain many properties of epithelial cells; in fact these cells grow to confluence as a monolayer with the basolateral membrane attached to the surface of the culture dish and a microvillus-studded apical membrane facing the media, maintaining the polarity of the cell.

These intestinal epithelial cells are attached to each other and to the basement membrane through the interaction of adhesion molecules and junctional proteins such as tight junctions and desmosomes. The integrity of cellular adhesion determines the ability of an intestinal epithelial monolayer to serve as a barrier to luminal molecules. These cells also respond to

a variety of peptides, hormones and neurotransmitters in a manner similar to isolated intestine, indicating the presence of receptors and transport processes. This cell line, specifically responds to p31-43 with induction of actin reorganization and stress fiber formation (Maiuri et al., 2005).

In the present study I observed that differences in actin reorganization between cells treated with the peptide p31-43 and (control) untreated cells, were discernible within 15 minutes of peptide exposure. Treated cells showed an increase in fine actin cables within the cell body. By 20 minutes of peptide p31-43 treatment, these morphological differences were much more pronounced. These changes were followed by thickening of actin bundles and the subsequent increase in the number and length of F-actin stress fibers traversing the cell (**Fig 3.1.b).** In untreated cells F-actin was concentrated along the cell borders and was generally absent in the central region of the cell (**Fig 3.1.a).** As control, in all the experiments T84 cells were stimulated with the immunodominant peptide pα-2 or pα-9 (data not shown). No effect on actin polymerization was observed.

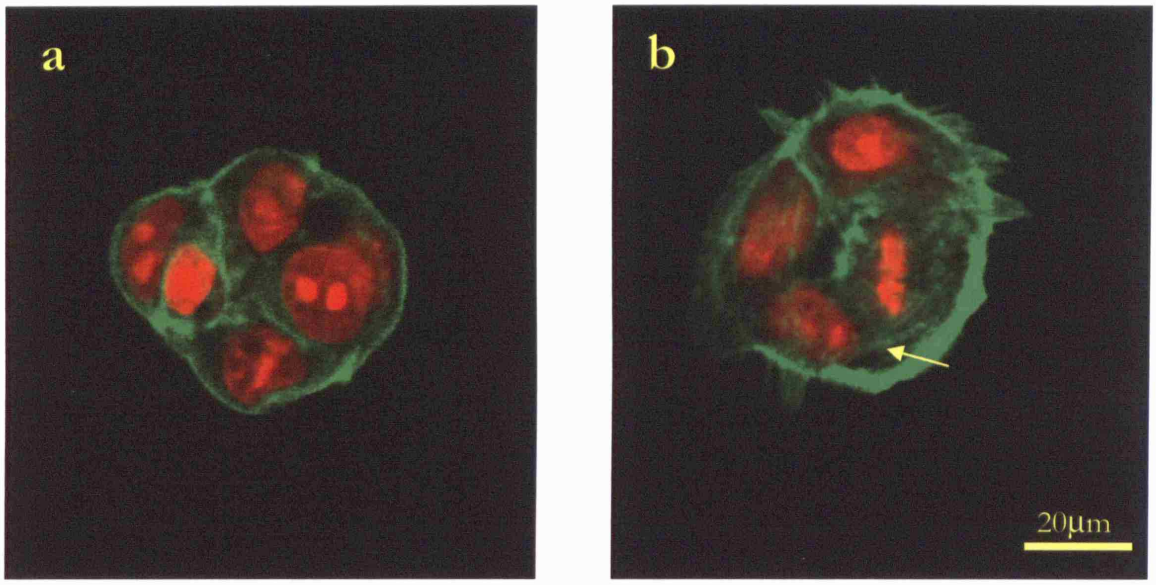


Figure 3.1 – Gliadin peptide p31-43 causes an increase in F-actin stress fibers formation. F-actin rearrangement in intestinal epithelial cells line T84. T84 cells were treated in vitro with medium alone (3.1.a) and gliadin peptide 31-43 for 20 minutes (3.1.b). Arrow shows stress fibers formation. F-actin (green) was visualized with Phalloidin FITC. Nuclei (red) were stained with TO-PRO. The experiment was repeated 5 times and all the data reported in this figure were reproduced in the 5 separate experiments.

3.2.2 Epithelial modifications are dependent on actin polymerization

Next I investigated whether these modifications induced by p31-43 were dependent on cytoskeletal reorganization. To understand whether actin polymerization was important in the cell modifications induced by the gluten peptide, T84 cells were pretreated with the actin filament disrupting agent Cytochalasin D, prior to stimulation of cells with p31-43.

Cytochalasin D is a cell-permeable fungal toxic metabolite which inhibits actin polymerization by binding to growing ends of actin nuclei and filaments (F-actin), and preventing addition of monomers (G-actin) to these sites. After use of Cytochalasin D as depolymerizing agent, when the actin cytoskeleton was severely disrupted, before p31-43

treatment, no stress fibers formation was observed. These data suggest that an intact cytoskeleton in intestinal epithelial cells would appear to be necessary for the modifications induced by p31-43 (**Fig 3.2**).

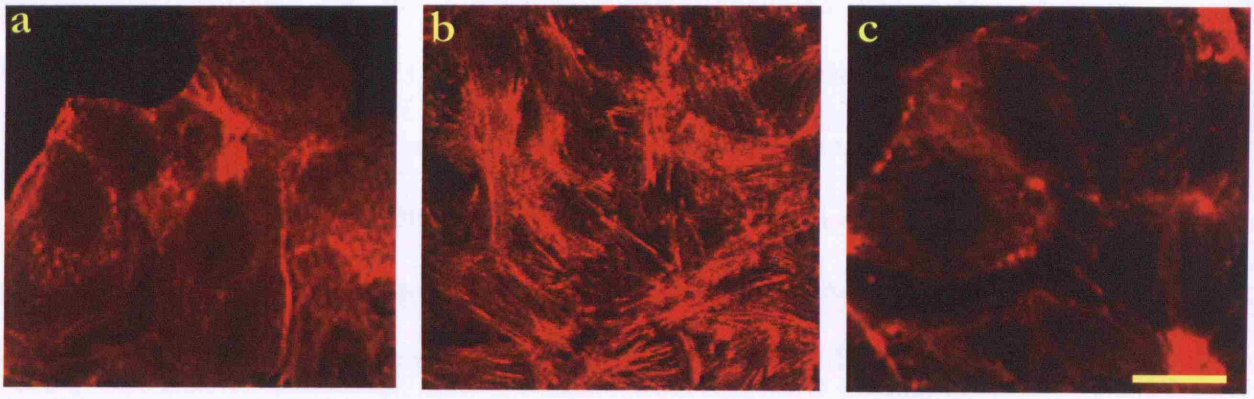


Figure 3.2 - Intact cytoskeleton in T84 cells is necessary for gliadin peptide p31-43 induced modifications. T84 cells were not stimulated (3.2.2.a), treated with p31-43 for 20 minutes (3.2.2.b), and pre-treated with Cytochalasin D before p31-43 treatment (3.2.2.c). F-actin (red) was visualized with Phalloidin TRITC. The experiment was repeated 5 times and all the data reported in this figure were reproduced in the 5 separate experiments. Bar shows 20µm.

3.2.3 Rho-A induces actin modifications in epithelial cells

To explore whether Rho-A GTPase was involved in this actin reorganization and was required for the cellular responses induced by gliadin, T84 cells were microinjected with an inhibitor of endogenous Rho-A before addition of the gliadin peptide p31-43.

T84 cells were microinjected with a plasmid encoding specific Rho-A inhibitor C3 transferase from *Clostridiun botulinum* that has been shown to inhibit Rho by adenosine diphosphate (ADP)-rybosylation of this protein. This construct contained a Myc epitope

tag engineered at the amino terminus that allowed detection of the exogenous protein and identification of microinjected cells using a monoclonal antibody anti Myc (clone E910). The effectiveness of Rho-A inhibitor microinjected was demonstrated by its ability to specifically reduce Rho-A expression in T84 cells. Immunofluorescence analysis showed that microinjection with the Rho-A inhibitor markedly inhibited stress fiber formation induced by the gliadin peptide 31-43 (**Fig 3.3.b and c**). C3-injected cells remained well spread and did not form stress fibers. As a control cells were pretreated by microinjecting enhanced green fluorescent protein (EGFP) in the cells before p31-43 treatment, to check whether the stress fiber formation inhibition in T84 cells was a real effect of the Rho-A inhibition and also whether the microinjection process could influence the stress fiber formation. Microinjection of EGFP did not interfere with actin rearrangement and formation of stress fibers induced by the gliadin peptide (**Fig 3.3.d**). Therefore we could conclude that the Rho-A inhibition with the microinjection of C3 transferase was effective in inhibiting the stress fiber formation induced by p31-43 and also that microinjection itself did not inhibit stress fiber formation.

I also examined the role of Rho-A proteins in actin reorganization by pre-treating T84 cells with soluble Rho-A C3 transferase to further analyze the effect of Rho-A on other cellular modifications observed in the epithelium induced by gluten. C3 transferase significantly inhibited stress fiber formation induced by gliadin peptide 31-43 (**Fig 3.3.g**). These results indicate that the gliadin peptide p31-43 induced actin modification, with stress fiber formation, are highly sensitive to Rho-A neutralization stressing the role of this signal transduction pathway in immune innate activation by gliadin.

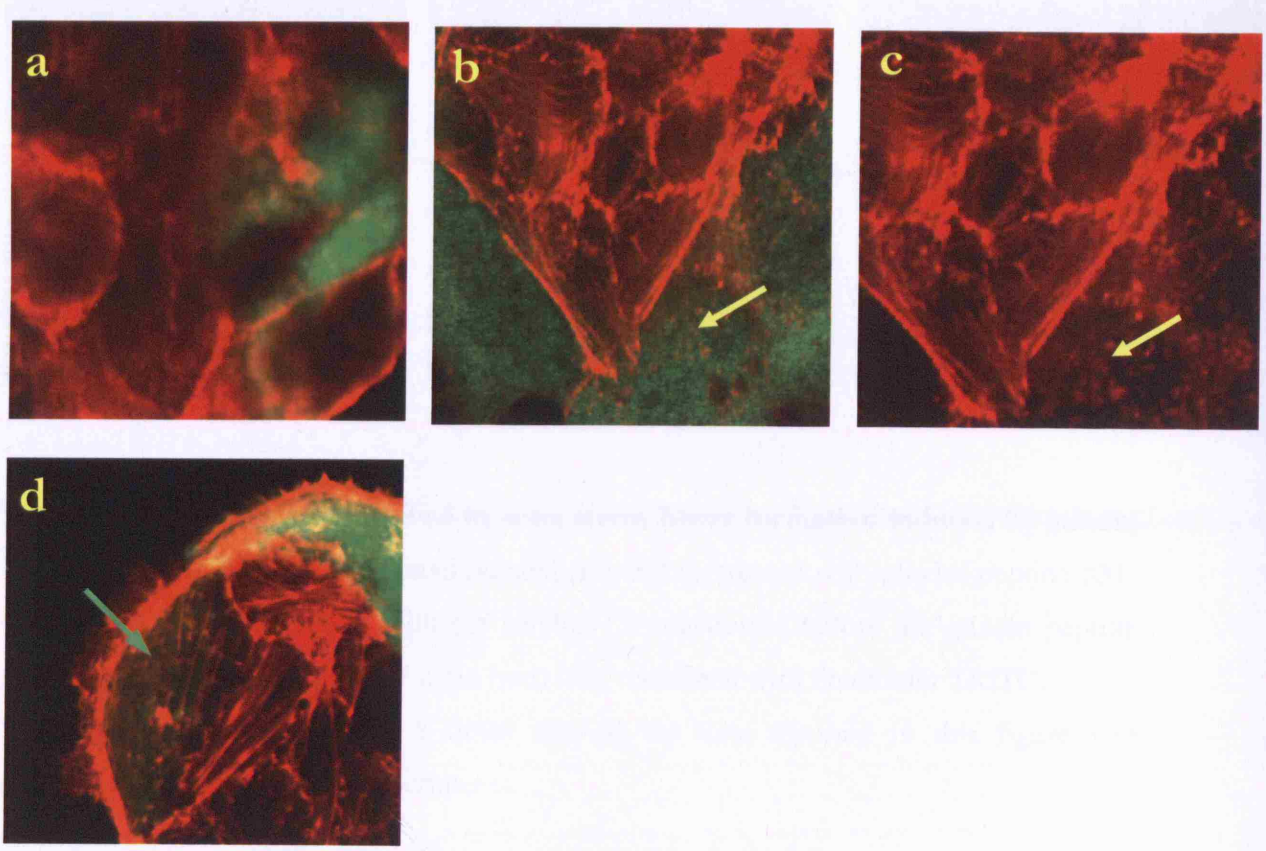


Figure 3.3 - Rho-A is involved in actin stress fibers formation induced by gliadin peptide 31-43. T84 cells were microinjected into the nuclei with plasmid encoding Rho-A Inhibitor, Myc-tagged C3 transferase (Fig 3.3.a, b and c). F-actin (red) was visualized with Phalloidin TRITC. Figures 3.3.a 3.3.b show cells stained with Phalloidin TRITC and antibody anti Myc (green) (to visualize microinjected cells). Figure 3.3.c shows the F-actin staining only.

Cells then were untreated (Fig 3.3.a) or treated with the gliadin peptide p31-43 (Fig 3.3.b and 3.3.c). Figure 3.3.d shows cells microinjected with a control plasmid encoding EGFP that were treated with gliadin peptide and stained with Phalloidin TRITC. The experiment was repeated 5 times and all the data reported in this figure were reproduced in the 5 separate experiments.

Figure 3.3 bis - Rho-A is involved in actin stress fibers formation induced by gliadin peptide 31-43. T84 cells were unstimulated (Fig 3.3.e), treated with gliadin peptide p31-43 (Fig 3.3.f) and pretreated with the soluble C3 transferase before the gliadin peptide p31-43 stimulation (Fig 3.3.g). F-actin (red) was visualized with Phalloidin TRITC.
The experiment was repeated 5 times and all the data reported in this figure were reproduced in the 5 separate experiments.

3.2.4 Rho-A induces protein tyrosine phosphorylation in epithelial cells

Actin stress fibers emanate from distinct areas of the plasma membrane known as focal adhesions, where clustering of integrin receptors bind to extracellular matrix proteins including fibronectin and collagen. Focal Adhesion Kinase (FAK) is a non receptor protein kinase that localises to focal contact sites and has been linked to the generation of cell survival, cell cycle progression, and cell motility signals (Schlaepfer et al., 1999).

It has previously been observed that one of the early events in CD, after challenge *in vitro* with gliadin peptide 31-43, is an increase in phosphorylation of proteins at tyrosine residues

on the enterocytes with specific up-regulation of phosphorylation at Tyr residues in Focal Adhesion Kinases.

Phosphorylated FAK at Tyr^{397} creates an SH2 binding motif (Schaller et al., 1994) that is required for FAK function in promoting cell motility (Sieg et al., 1999).

T84 cells are known to specifically respond to gluten p31-43 with induction of protein tyrosine phophorylation and also FAK phosphorylation.

Several lines of evidence suggest that protein tyrosine phosphorylation is important in the Rho signalling pathway (Ridley and Hall, 1994). Rho-A induces focal adhesions and concomitantly also tyrosine phosphorylation of a number of proteins, including focal adhesion kinases and these focal adhesions can be inhibited by tyrosine kinase inhibitors (Ridley and Hall, 1994), suggesting that protein tyrosine phosphorylation is required downstream of Rho in this signalling pathway.

I therefore investigated whether Rho-A could be involved also in this signalling process of Tyrosine phosphorylation induced by gliadin peptide 31-43. I found that an increase in the phosphotyrosine content of a number of proteins was observed 30 minutes after cells were treated with p31-43 gluten peptide as detected by specific antibodies against phosphoTyrosine (**Fig 3.4.b**). No such response was observed in control untreated cells (**Fig 3.4.a**) or when cells were treated with the immunodominant peptide p-α2 or p-α9. When T84 cells were pre-treated with Rho-A inhibitor C3 exoenzyme, a decrease of protein tyrosine phosphorylation was observed (**Fig 3.4.c**). I set out to identify these tyrosine phosphorylated proteins. One candidate for a downstream target of Rho was the Focal adhesion kinase phosphorylated at Tyr 397 which has previously been shown to be up-regulated by p31-43 gliadin peptide using a specific antibody anti FAK phosphorylated at

Tyr^{397} (**Fig 3.4.e**). I examined the effect of C3 exoenzyme on this signalling pathway with Tyrosine phosphorylation and FAK activation induced by gliadin peptide 31-43 on T84 cells. Pre-treatment with C3 exoenzyme markedly inhibited the peptide induced FAK phosphorylation at Tyr^{397} (**Fig 3.4.f**). These findings demonstrate that in T84 cells, Rho-A was able to induce the phosphorylation of focal adhesion kinases establishing that p31-43 induced tyrosine phosphorylation and FAK phosphorylation are Rho-A dependent responses.

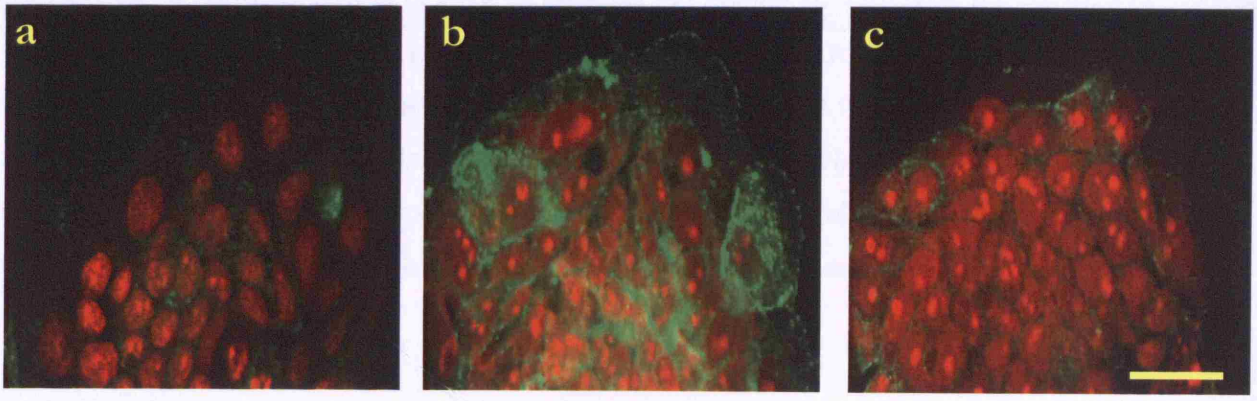


Figure 3.4 – Rho-A induces protein tyrosine phosphorylation in T84 cells.
T84 cells were untreated (Fig 3.4.a), treated with the gliadin peptide p31-43 for 20 min (Fig 3.4.b). An increase of phosphorylation of proteins at tyrosine level compared with cells not stimulated was observed. Pretreatment with the soluble C3 transferase followed by the p31-43 stimulation, prevented p31-43 induced tyrosine phosphorylation (Fig 3.4.c). T84 cells were stained with antibody anti phosphotyrosine (green). Nuclei were stained with Propidium Iodide (red).
The experiment was repeated 5 times and all the data reported in this figure were reproduced in the 5 separate experiments. Bar shows 10μm.

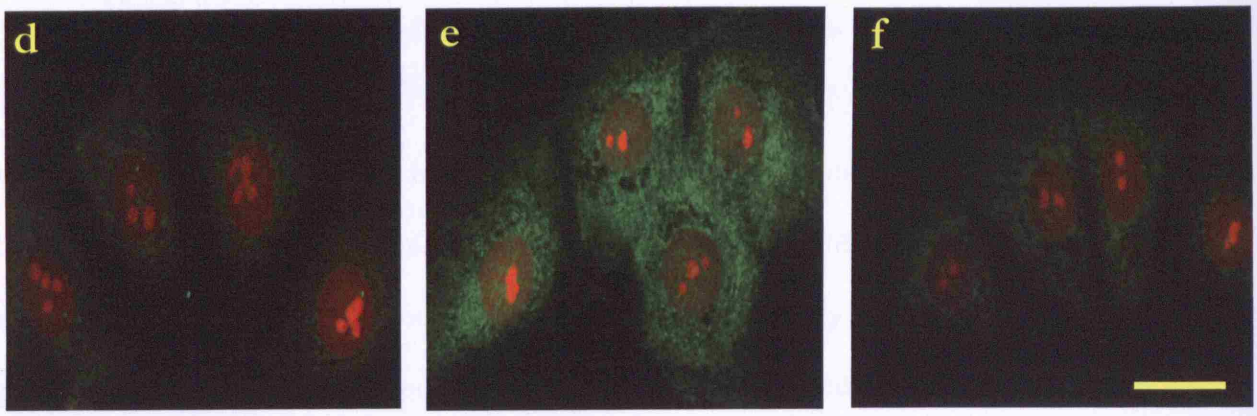


Figure 3.4 bis – Rho-A induces Focal Adhesion Kinase phosphorylation in T84 cells.
T84 cells were untreated (3.4.d), treated with the gliadin peptide p31-43 for 20 min (3.4.e). An increase of phosphorylation of FAK at tyrosine 397 compared with cells not stimulated was observed. Pre-treatment with the soluble C3 transferase followed by the p31-43 stimulation, prevented p31-43 induced phosphorylation of FAK at tyrosine 397 (3.4.f). T84 cells were stained with antibody anti phosphoFAK^{397} (green). Nuclei were stained with Propidium Iodide (red). The experiment was repeated 5 times and all the data reported in this figure were reproduced in the 5 separate experiments. Bar shows 20µm.

3.2.5 Gliadin peptide p31-43 induces apoptosis in epithelial cells T84 through a Rho-A downstream effector ROCK-1.

Cell death is a vital part of life in multicellular organisms, playing roles in development, defence and homeostasis. In animals, most cell death is via the process of apoptosis, especially when cell death proceeds as part of normal physiology. Apoptosis, also called programmed cell death, is an important and very carefully regulated process that allows the multicellular organisms to remove cells selectively. This removal may be because cells are in excess, because they are infected or damaged, because they have failed a developmental "test" or because their presence is no longer needed for a physiological process. The coordination and balance between cell survival and apoptosis is crucial for normal development and homeostasis of multicellular organisms. Defects in control of this balance may contribute to a variety of diseases, including cancer, autoimmune disease and neurodegenerative conditions. It is a form of "cell suicide" and although induced by a wide range of signals, is a tightly controlled process occurring in a regulated and sequential manner. The apoptotic process is characterized by changes in cellular morphology including cell shrinkage, chromatin condensation and pyknosis, along with biochemical events leading to loss of mitochondrial membrane potential, loss of plasma membrane asymmetry that is contorted into "blebs" and DNA cleavage between nucleosomes. The appearance of phosphatidyl serine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell creates one of the specific signals for recognition and removal of apoptotic cells by macrophages and other phagocytosing cells. Apoptosis is orchestrated by the action of a set of proteases in the cells called caspases (cysteine proteinases with specificity for aspartate residues). When caspases are activated, they

cleave specific substrates, either activating or inactivating them. These produce the morphologic and phagocitic changes associated with apoptosis. Caspases activate enzymes that cause cellular changes, including DNA fragmentation and the externalization of phosphatidylserine (PS) on the plasma membrane. There are two general categories of caspases involved in apoptosis that are distinguished by their chemistry and their function. The "executioner" caspases (caspase-3, -6 and -7) exist as inactive dimers in their proforms and reside predominantly in the cytosol. They are activated by cleavage at specific sites generating the large and small subunits in the mature enzymes. The major enzymes responsible for cleaving and activating the executioner caspases are the "initiator" caspases. Unlike the executioner caspases, the initiator caspases cannot be activated by cleavage (such cleavage occurs, but this does not cause the formation of an active site). Initiator caspases are caspase-8 and -9. The initiator caspases exist in their proforms as monomers, and it appears that they can only be activated by their dimerization (Green, 2003). Apoptosis is crucial for the maintenance of the epithelial functions of the gut as it is involved in the normal epithelial cells turnover.

The intestinal villi consist of differentiated absorptive cells that originate from stem cells within the intestinal crypts. Epithelial cells in fact undergo a relatively rapid generation and death throughout the life of the organism. The cells are derived from stem cells located in the middle of the crypt. Asymmetric division of stem cells is essential to insure maintenance of stem cell number and final homeostasis of the intestinal epithelium. The cellular position along the crypt-villus unit varies with the differentiation state of its cells (bottom for undifferentiated cells, and top for more differentiated cells) (de Santa et al., 2003).

Under physiological conditions apoptotic cells are restricted to the tips of the villi in the small bowel and are replaced by an equal number of proliferating immature crypt cells (Watson, 1995).

In immune-mediated disorders such as celiac disease, an increased number of enterocytes undergo premature apoptosis all along the crypt-villous axis (Moss et al., 1996). The combination of increased apoptosis and altered turnover rates results in architectural changes in the celiac mucosa, characterized by villous atrophy and crypt hyperplasia.

Several studies support the hypothesis that wheat gliadin displays a direct cytotoxic activity against enterocytes (Giovannini et al., 2003; Maiuri et al., 2001).

It has been observed that the gliadin peptide p31-43 induces apoptosis of epithelial cells of celiac mucosa. Similar findings are evident in the transformed epithelial cell line T84.

Recent research has revealed the importance of actin cytoskeleton and the signal transduction pathway controlled by the Rho GTPases in regulating the changes in cell morphology observed in the process of apoptosis (Coleman and Olson, 2002) I therefore set out to investigate which signalling pathway was involved in the p31-43 induction of cell death in T84 cells.

To visualize apoptotic cells I used Annexin V, which has a high affinity for binding to PS, conjugated with FITC. As the apoptotic process progresses, cell membrane integrity is lost and DNA is fragmented. Hence by using Propidium Iodide (PI), that is a DNA-specific viability dye, I was able to distinguish between early apoptotic, late apoptotic and dead cells.

I therefore set out to establish whether there was a direct role of cytoskeletal rearrangement in p31-43-induced cell death of epithelial cells since in other models (i.e. CD47 induced

apoptosis (Mateo et al., 2002)), polymerised actin is established to be important in inducing PS exposure and membrane blebbing leading to apoptosis.

T84 cells were cultured *in vitro* for 24 h with medium alone or with the addition of p31-43 peptide. As control in all experiments T84 cells were treated with the immunodominant peptides pα-2 or pα-9 (data not shown)

I pre-treated T84 cells with the actin polymerization inhibitor, Cytochalasin-D, before the 24h of gluten peptide p31-43 stimulation (**Fig 3.5.1** and **3.5.2**). The expression of the percentage of apoptotic cells was quantified by flow cytometry. I did not observe any inhibition of apoptosis induced by p31-43 in T84 cells after CytochalasinD pretreatment (**Fig 3.5.1.c**). No effect on the apoptotic cells was observed in fact comparing with cells treated with the peptide alone (**Fig 3.5.1.b**). Percentage of apoptotic cells after the different treatments *in vitro* is also shown graphically in **Fig 3.5.2**. All these data taken together suggest that there is not a direct role of actin cytoskeleton in p31-43-induced apoptosis.

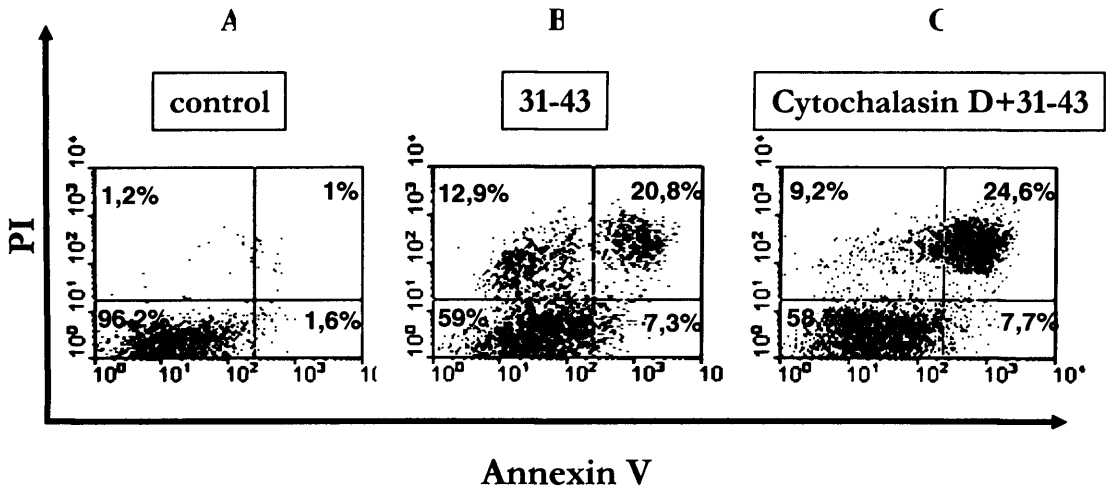


Figure 3.5.1 – Integrity of actin cytoskeleton does not have a direct role in gliadin peptide p31-43 induced apoptosis in T84 cells.

T84 cells were cultured in medium alone (control), treated with p31-43 for 24 h, or pretreated with F-actin polymerization inhibitor, Cytochalasin D, before stimulation with p31-43 for 24h. Pretreatment of T84 cells with Cytochalasin D before the 24h p31-43 stimulation does not have any effect on the apoptosis p31-43. Percentage of apoptotic cells is represented by positive staining with Annexin V and PI. The experiment was repeated 5 times and one out of five independent experiments is shown.

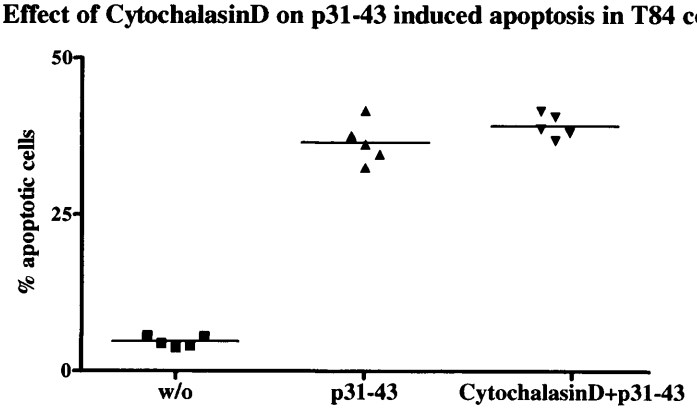


Figure 3.5.2

CytochalasinD does not decrease the percentage of apoptosis in T84 cells induced by peptide p31-43. Statistical significance was analysed by Student's *t*-test.

I then investigated whether Rho-A was involved in this process (**Figure 3.6.1** and **3.6.2**). Apoptosis induction was only minimally decreased (**Fig 3.6.1.c**) by Rho-A inhibition but the reduction of the percentage of apoptotic cells was not significative compared with cells treated with p31-43 alone. These results suggest that other pathways are involved in p31-43 induced cell death.

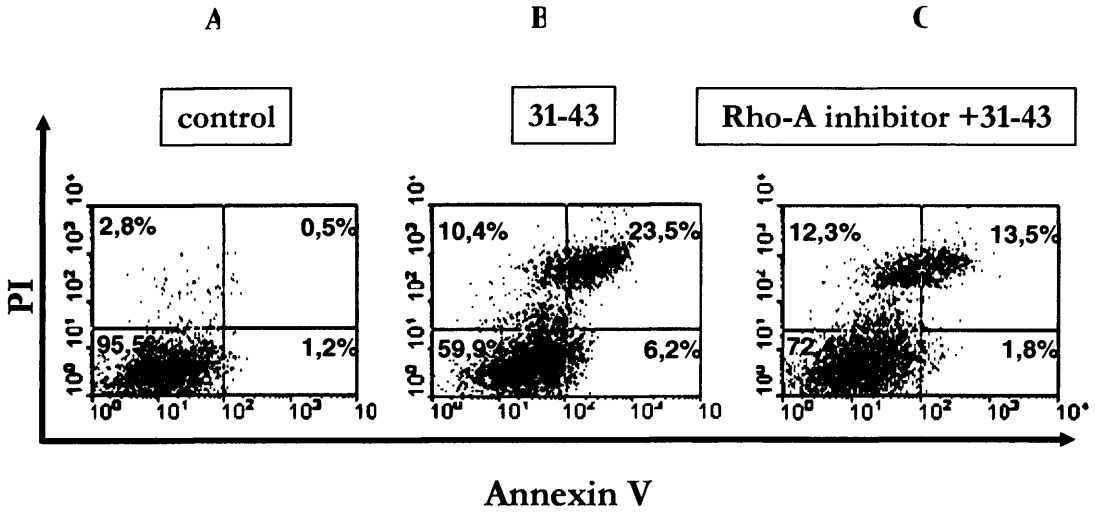


Figure 3.6.1 – Apoptosis in T84 cells is moderately controlled by Rho-A.

T84 cells were cultured in medium alone (control), treated with p31-43 for 24h, or pretreated with Rho-A inhibitor, C3 transferase, before stimulation with p31-43 for 24h . Pretreatment of T84 cells with C3 before the 24h p31-43 stimulation moderately inhibits the apoptosis induced by p31-43. Percentage of apoptotic cells is represented by positive staining with Annexin V and PI. The experiment was repeated 5 times and one out of five independent experiments is shown.

Effect of Rho-A inhibitor on p31-43 induced apoptosis in T84 cells

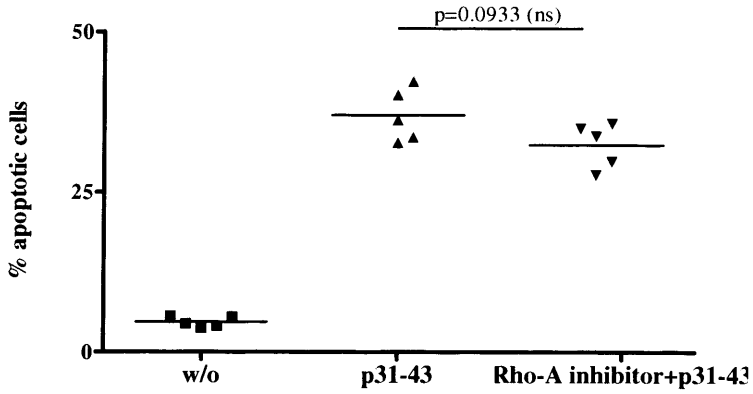


Figure 3.6.2. Rho-A inhibition does not significantly decrease the percentage of apoptosis in T84 cells induced by p31-43. Statistical significance was analysed by Student's *t*-test.

A better inhibition of the apoptosis was observed when cells were pre-treated with an inhibitor of the downstream Rho-A effector, the Rho-A kinase I, ROCK-I (**Fig 3.7.1** and **3.7.2**). The reason why ROCK-I resulted in a better apoptosis inhibition (**Fig 3.7.c**) might be due to the fact that ROCK-I can be activated also by alternative pathways leading to cell death such as the Caspase-3 mediated pathway (Coleman and Olson, 2002).

Following the induction of apoptosis, p31-43 induces caspase-3 activation that cleaves ROCK-1, removing its inhibitory domain and thereby producing an active truncated kinase. ROCK-1 controls actomyosin filament assembly by phosphorylation of myosin regulatory light chain (MLC) that subsequently stimulates increased contractility, blebbing and apoptosis.

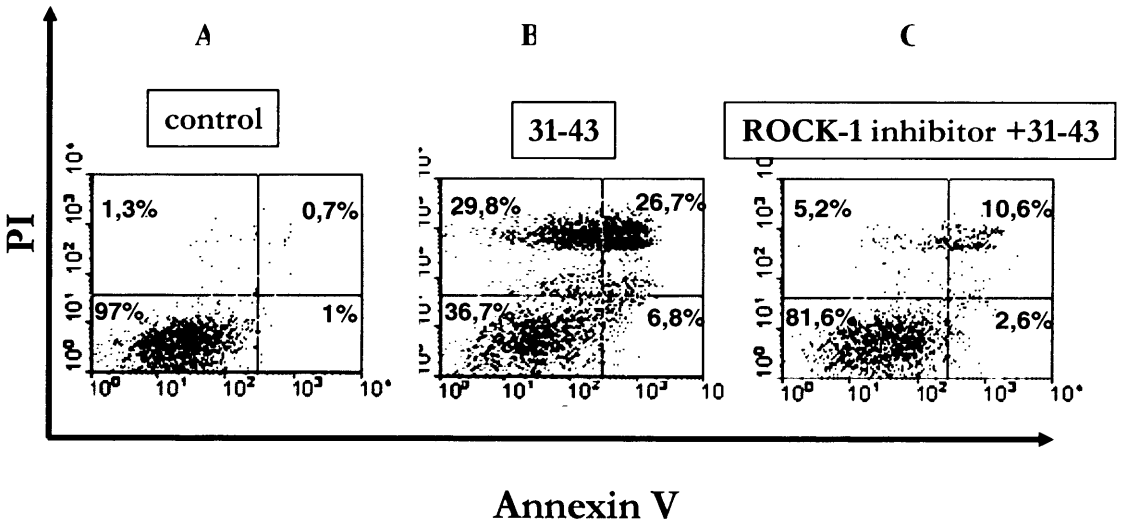


Figure 3.7 - Rho-A downstream effector ROCK-1 induces apoptosis in epithelial cells. T84 cells were cultured in medium alone (control), treated with p31-43 for 24 h, or pretreated with ROCK-I inhibitor, Y-27632, before stimulation with p31-43 for 24 h. Pretreatment of T84 cells with ROCK-I inhibitor before the 24h p31-43 stimulation inhibits the apoptosis induced by p31-43. Apoptotic cells are represented by positive staining with Annexin V and PI. The experiment was repeated 5 times and one out of five independent experiments is shown.

Effect of ROCK-1 inhibitor on p31-43 induced apoptosis in T84 cells

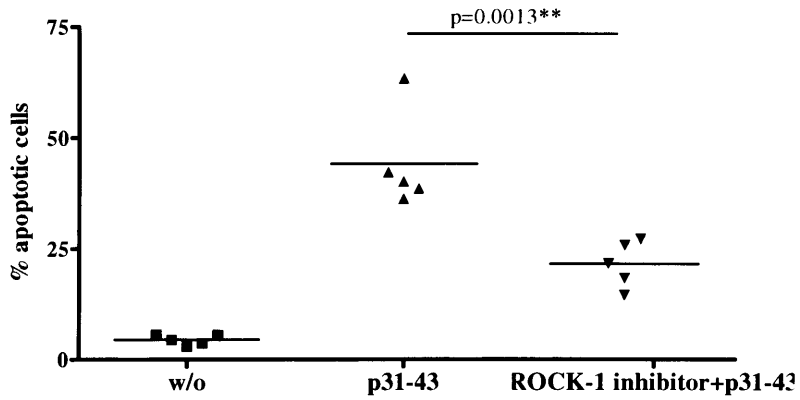


Figure 3.7.2

ROCK-1 is involved in the apoptotic pathway induced by the gliadin peptide p31-43.

The percentage of apoptosis induce in T84 cells by the peptide p31-43 is significantly decreased by the inhibitions of Rho-A downstream effector ROCK-1. Statistical significance was analysed by Student's *t*-test.

3.3 Discussion

One of the early modifications that gluten induces in celiac patients enterocytes, is actin filament reorganization followed by epithelial modifications leading to apoptosis. This is considered to be at the basis of celiac disease pathogenesis. These results, performed with the intestinal epithelial cell line T84, indicate that gliadin peptides induce actin modifications, with stress fiber formation. This process is highly sensitive to Rho-A neutralization stressing the role of this signal transduction pathway in immune innate activation by gluten. The cytoskeletal modifications observed in the intestinal epithelial cells induced by the innate gliadin peptide p31-43 produced a signal pathway through Rho-A activation. In particular, tyrosine phospshorylation and actin reorganization, are highly sensitive to Rho-A neutralization, stressing the role of this signal transduction pathway in the innate activation mediated by gliadin.

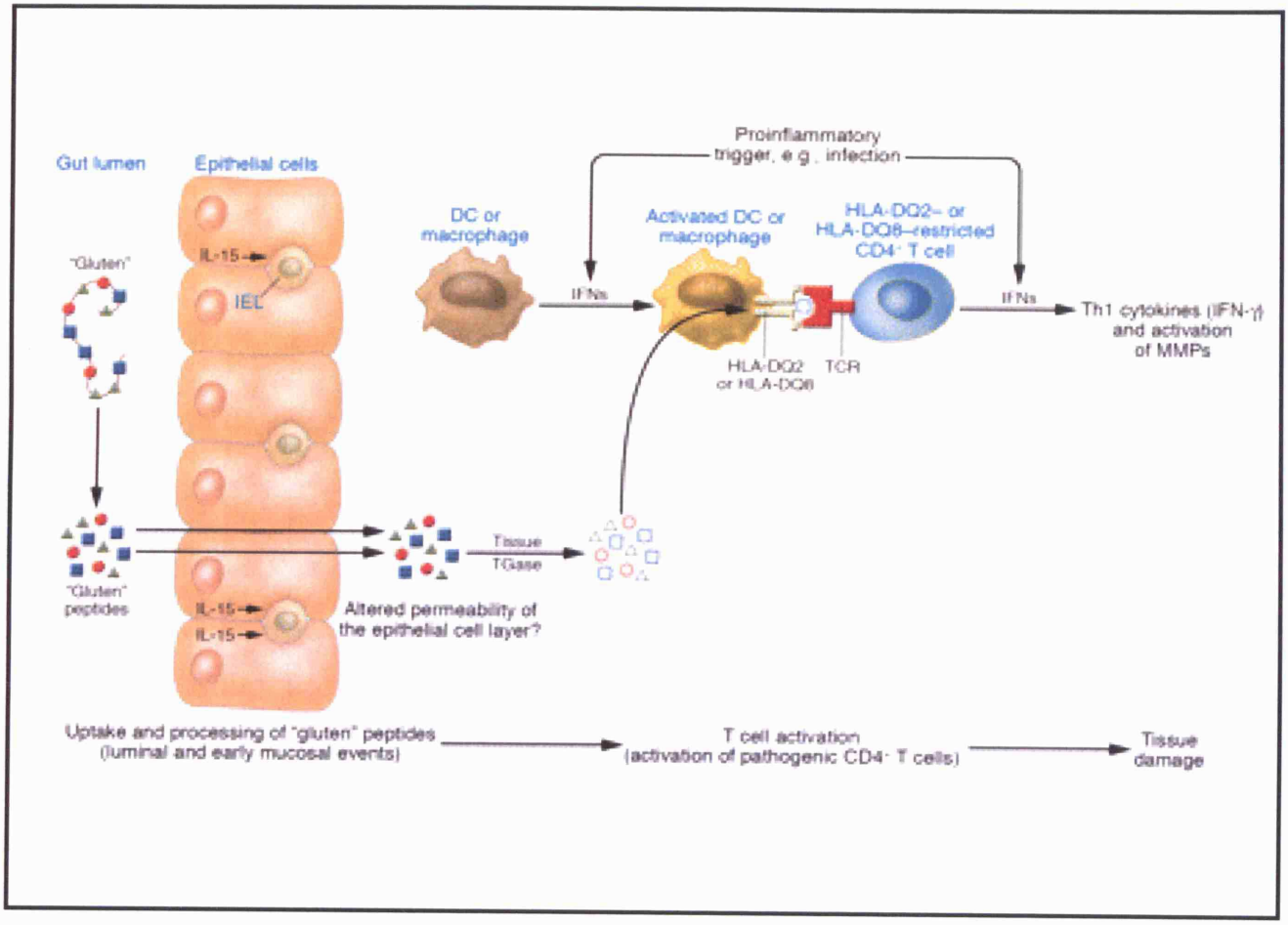
ROCK-I inhibition, a downstream effector of Rho, significantly controlled epithelial apoptosis whilst Rho inhibition failed to do so.

These data support a central role of the Rho-A signal transduction pathway in gliadin induced epithelial modification in Celiac Disease.

Chapter 4 – $CD4^+CD25^+FOXP3^+$ Regulatory T cells in the immune response to gluten in Celiac Disease (CD).

4.1 Introduction

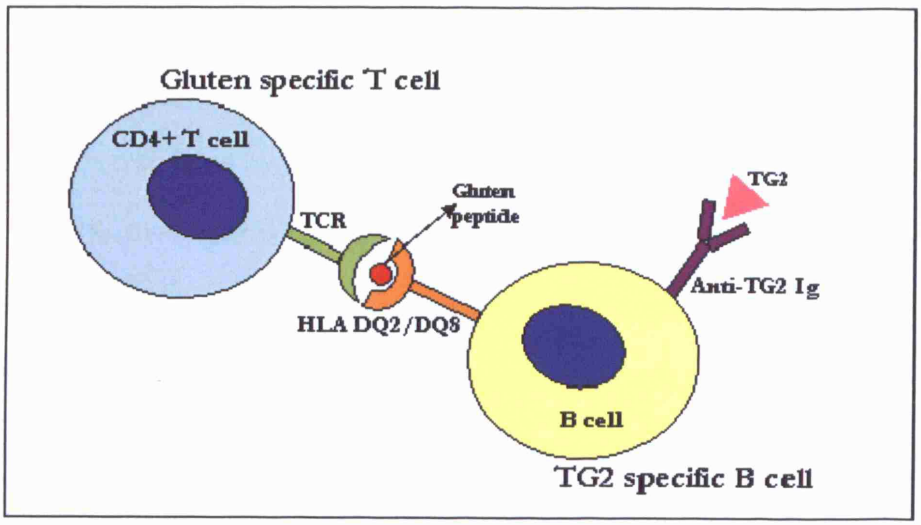
In Celiac Disease (CD) a break-down in immunological tolerance against gluten is observed. This immune response is prevalently $CD4^+$ T cell mediated. Gluten, because of its high proline content, is incompletely digested within the intestinal lumen, giving rise to gluten peptides. These peptides cross the epithelial barrier to the lamina propria and encounter tissue transglutaminase (tTG). tTG deamidates the peptides so that they expose negatively charged amino acids such as glutamic acid which are recognized with high affinity by APCs that express HLA DQ2/DQ8 heterodimers. These gluten peptides, once deamidated by tTG, are presented by APCs to HLA DQ2/DQ8-restricted populations of pathogenic $CD4^+$T cells in the lamina propria that become activated and release mediators (cytokines) that lead to inflammation and ultimately to tissue damage (Kagnoff, 2007). tTG it is not simply the "modifier" enzyme that transforms gluten peptides to induce an immunological response; in addition it becomes an auto-antigen being recognized as non-self by the IgA and IgG auto-antibodies anti tissue-transglutaminase. The production of anti tTG auto-antibodies is likely to be dependent on cognate T cell help to facilitate isotype switching of autoreactive B cells (Sollid and Jabri, 2005).



Kagnoff MF, *JCI*, 2007.

Figure 4.1 - Pathogenesis in CD mucosa

In CD it is observed the activation of both the innate and adaptive immune response. Firsltly gluten induces an early response with the activation of epithelial cells, secretion of IL-15 (innate cytokine) with the activation of dendritic cells or macrophages that set the adaptive immune response in the lamina propria.



Sollid, *Curr Opin Immunol*, 2005.

Figure 4.2 Mucosal antigens in CD

In CD the adaptive immune response is T cell mediated with the activation of gluten specific T cells and B cell mediated with the activation of B cells producing antibodies for the auto anrigen tissue transglutaminase.

A mechanism of immune regulation in the small intestine of individuals genetically predisposed to develop CD is really important to balance the immune response between mucosal antigens such as gluten, and pathogenic immune cells to keep immunological tolerance and to prevent an autoimmune response. A phenomenon known as oral tolerance occurs in response to mucosal antigens and establishes a mechanism of regulation that ensures mucosal homeostasis so that most mucosal responses are self-limited and do not result in inflammation (Strober et al., 1998). The mechanisms responsible for normal intestinal homeostasis in the presence of harmless antigens such as gluten are not well understood (Faria and Weiner, 1999).

Functionally distinct subsets have been clearly defined amongst $CD4^+$T cells which are of importance in maintaining immune tolerance. Among these subsets, the key type of cells involved in controlling the magnitude and type of the immune response is represented by the subset of naturally occurring regulatory T cells. T-regulatory cells (Tregs) down-regulate immune responses by suppressing autoreactive T cells for both foreign and self-antigens and effectively participate in the control of autoimmune disorders (Sakaguchi, 2005) by maintaining immunological self-tolerance and negative control of pathological as well as physiological immune responses (Shevach, 2002). Phenotypically naturally occurring regulatory T cells are defined by the co-expression of $CD4^+$ and the α chain of the interleukin-2 Receptor, $CD25^{+high}$. Other markers may also identify these regulatory T cells including the transcription factor FOXP3 that is a 'master control gene' for the development and function of these cells. They are produced by the normal thymus as a functionally distinct and mature population. Treg cells are essential for the maintenance of self tolerance and their activation through oral antigen administration suggests that these cells may also be involved in oral tolerance. Treg cells have been shown to be able to significantly control gut inflammation in mouse models of IBD as transfer of $CD4^+CD25^+$ T reg cells can prevent colitis (Mottet et al., 2003). In these studies $CD4^+CD25^+$ T cells can reverse an established T cell mediated inflammatory response in the intestinal mucosa by reducing the pathogenic T cell infiltrate, ultimately leading to restoration of normal intestinal architecture. In contrast to the mouse, in whom they are well characterized, relatively little is known about regulatory T cells in the human gut.

It is not known whether $CD4^+CD25^+FOXP3^+$Treg cells play an important role in the maintenance of mucosal homeostasis in CD but the ability of these cells to resolve

established inflammation in model systems raises the possibility that these cells may be useful as therapeutic agents for chronic inflammatory diseases of the gut in humans.

CD provides a convenient model in which to study the auto-inflammatory response as this can be convenient by the application of exogenous gluten.

In addition to the thymic production of natural $FOXP3^+$ Treg cells, naïve T cells in the periphery acquire FOXP3 expression and Treg function after chronic antigen stimulation *in vivo* (Apostolou and von, 2004). The ability to induce a Treg cell population from the naïve pool may be of particular benefit in the intestine where the immune system must cope with the challenge of an extremely high load of antigens and also with self-antigens, as gluten in CD, recognised as non self. It may therefore be crucial to generate Treg cells specific for this antigen, in addition to those Tregs selected for their high affinity to self antigens in the thymus to keep the immune response against gluten at bay.

Therefore, in this study I have examined whether Treg cells were present in CD mucosa and also whether they had any role in the regulation of the immune response driven by gluten stimulation. An approach to identify the presence of a regulatory defect in inflammatory disorders of the gut is to characterize the possible regulatory cells that are present in the lesion and in peripheral tissues of these patients. In previous studies in CD it has been reported that incubation of CD mucosa with gliadin (alcohol soluble portion of gluten) induces an increase of $CD4^+CD25^+$ cells in lamina propria cells (Lundin KE, 1993). Herein, during my study I have firstly investigated whether these $CD4^+CD25^+$cells in CD mucosa were Tregs by looking at the transcription factor FOXP3 expression. Identification of FOXP3 as a more specific marker for Tregs cells provides an opportunity to track the fate of Tregs *in vivo*. I have also tried to understand whether disease activity in CD

correlates with changes in frequency of Treg cells and their distribution in intestinal mucosa of CD patients. To study T regs in CD mucosa I have cultured *in vitro* the mucosa of CD patients and controls with gliadin to reproduce a situation "*ex vivo*" that could reproduce the actual situation *in vivo*. Gliadin was extracted from pure hexaploid bread wheat (Triticum aestivum variety San Pastore) and submitted to peptic-tryptic digestion. The peptic-tryptic digestion simulates the *in vivo* protein digestion by trypsin (protease secreted into the small intestine by the pancreas) and pepsin (protease secreted by gastric glands of the stomach into the stomach) to break-down dietary proteins to their components (peptides and amino acids) which can be readily absorbed by the small intestinal lining.

At the beginning of my study I had a look at the T regs as a percentage of CD4+CD25+FOXP3+ cells by isolating these cells from the intestinal lamina propria and analyse the data by flow cytometry.

The data obtained showed that FOXP3 was expressed in all the CD4+CD25+ population isolated. Unfortunately, due to the small size of the samples the number of T reg cells obtained was not sufficient to get enough conclusions and from that the decision to use FOXP3 as sole marker to identify Tregs in the mucosa.

The aim of the study reported in this chapter was to understand firstly whether $FOXP3^+$ cells were present in CD and also whether these cells had any regulatory role in suppressing the immune mediated response against gliadin.

4.2 Results

4.2.1 Patients with CD have an increased number of FOXP3$^+$ T cells in mucosal lymphoid tissue compared with controls.

To understand whether Tregs were present in CD mucosa and also whether these cells had any role in the disease pathogenesis I firstly tested the hypothesis that during active phases of the intestinal inflammation in CD the frequency of FOXP3$^+$ expression in nuclei of mucosal T cells was at variance with that observed in non inflammatory (control) tissues. Small intestinal mucosa biopsies from celiac patients and from controls were compared. To determine the localization of FOXP3$^+$ cells I performed immunofluorescence analysis.

FOXP3 expression in nuclei of Lamina Propria cells was determined, using specific monoclonal antibodies. I found that in tissue samples from non-inflamed controls and in patients with CD, FOXP3$^+$ nuclei were present in the lamina propria where T cells are mainly CD4$^+$.

The total number of cells expressing FOXP3+ was greater in the lamina propria of CD patients (with mean values: 7.250 ± 0.9520 N= 10) than in controls (with mean values: 3.667 ± 0.8819 N=6) **(Fig 4.3 and 4.4)**.

These data indicate that patients with CD have a higher expression of FOXP3$^+$ T cells than control subjects.

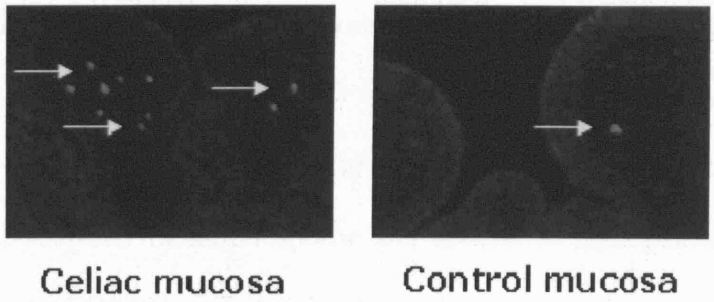


Figure 4.3 - FOXP3$^+$ protein expression is increased in nuclei of CD mucosa compared with controls. FOXP3$^+$ cells within small intestine of Celiac and control mucosal explants were analysed by immunofluorescence. Arrows show nuclear transcription factor FOXP3 expression. The number of FOXP3$^+$ cells is greater in CD mucosa than in control. Original magnification X 100. The experiment was repeated 5 times for each patient and control and the data were reproduced in the 5 experiments.

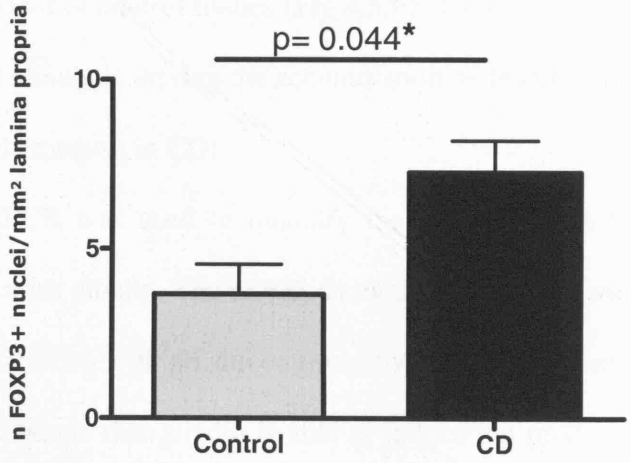


Figure 4.4 - FOXP3$^+$ nuclei expression in Lamina propria of small intestine mucosa from controls and from CD patients. The quantification is based on the number of FOXP3$^+$ positive nuclei per area (mm^2) of lamina propria. FOXP3+ cells within the lamina propria were counted in 10 random hpf. Statistical significance was analysed by Student's *t*-test. Plots show mean values with SD bars.

4.2.2 FOXP3 expression is up-regulated in celiac mucosa after challenge *in vitro* with gliadin.

To understand whether gliadin, as a specific antigen recognized by $CD4^+$Tcells in CD, which induces the adaptive immune response and leading to pathogenesis of the disease, was able to generate $FOXP3^+$ cells from naïve cells in the small intestine mucosa and induce an increase in their number and in their density in the lamina propria, I cultured *in vitro* mucosal samples from CD patients and controls with gliadin for 24h. The frequency of $FOXP3^+$ cells was estimated before and after *in vitro* stimulation with gliadin. The results show that the number of $FOXP3^+$ cells was greater in CD mucosa cultured *in vitro* with gliadin (with mean values: 11.69 ± 1.434 N=7) than in biopsies cultured in medium alone (with mean values: 7.000 ± 0.7786 N=7) **(Fig 4.5.a)**. This accumulation of $FOXP3^+$ cells after gliadin challenge was observed only in the lamina propria of celiac mucosa **(Fig 4.5.a)** and not in that of control tissues **(Fig 4.5.b)**. These data may suggest that the immune response against gliadin is driving the accumulation of $FOXP3^+$ cells in the effector sites of the lamina propria mucosa in CD.

In addition RT-PCR was used to quantify the level of FOXP3 mRNA in CD mucosa cultured in vitro with gliadin. The results show that FOXP3 transcripts are increased in CD mucosa cultured *in vitro* with gliadin compared with unstimulated mucosa **(Fig 4.6).**

These data demonstrate that gliadin is able to induce the production of $FOXP3^+$ cells at a peripheral level in the small intestine of CD patient's *ex-vivo*.

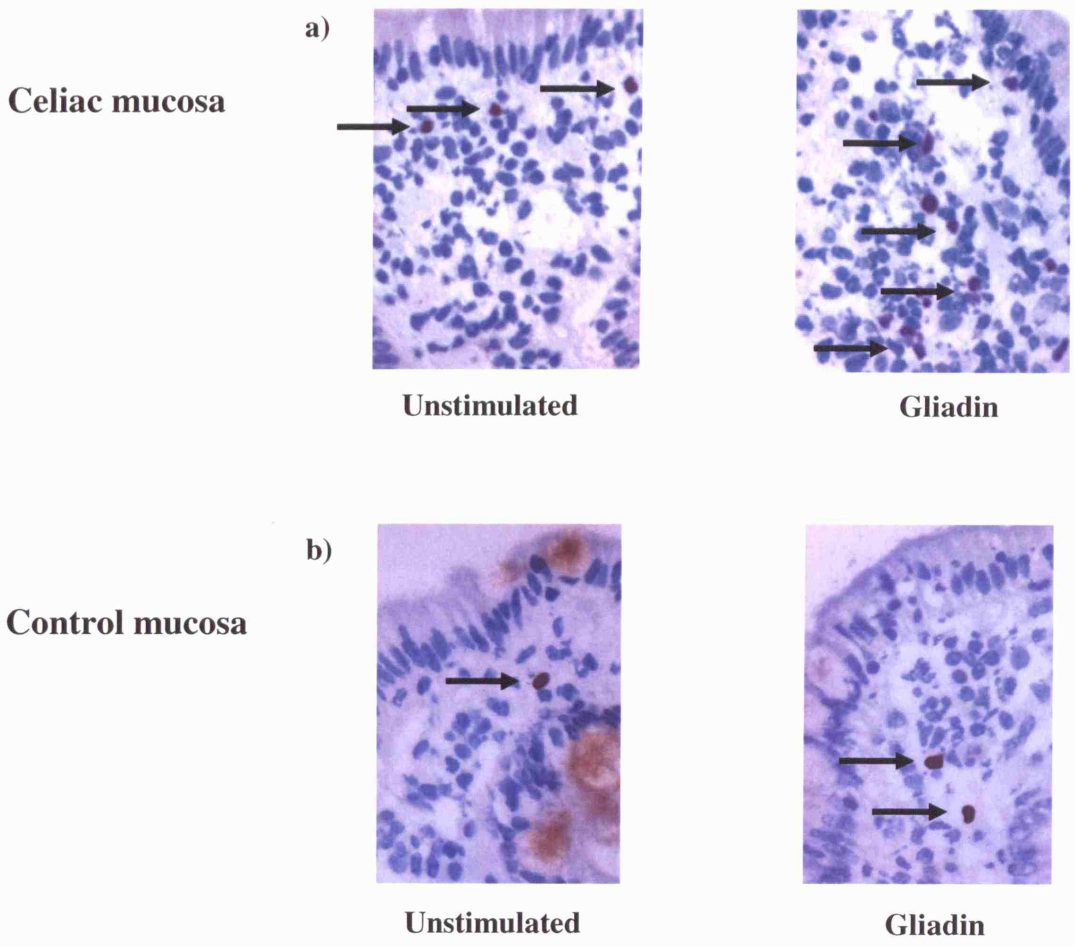


Figure 4.5.a and b FOXP3 expression is up-regulated in celiac mucosa after challenge *in vitro* with gliadin compared with controls. Celiac **(4.5.a)** and control **(4.5.b)** mucosal explants were cultured in vitro with medium alone (unstimulated) and with peptic–tryptic digest of gliadin for 24h. The transcription factor FOXP3 staining was visualized by immunoperoxidase. Nuclei were counterstained with Haematoxylin. Arrows show nuclear transcription factor FOXP3 expression. FOXP3 is up-regulated merely in CD mucosa after gliadin challenge and not in controls. Original magnification X 200. The experiment was repeated 5 times for each patient and control and the data reported in this figure were reproduced in the 5 separate experiments.

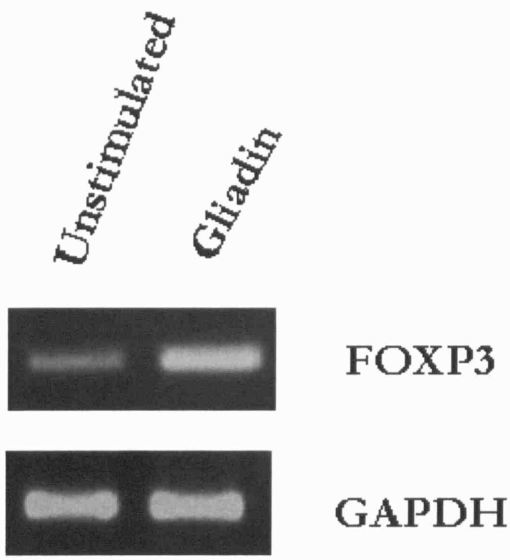
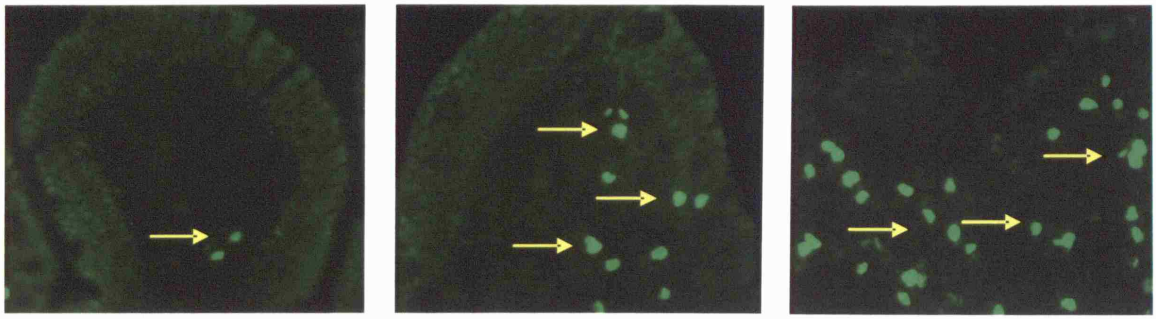


Figure 4.6 Transcription of FOXP3 is up-regulated in celiac mucosa after challenge *in vitro* with gliadin. Biopsy specimens from 6 celiac patients were cultured in vitro with medium alone (unstimulated) and with peptic–tryptic digest of gliadin for 6h. mRNA isolated from intestinal biopsies was subjected to RT-PCR and analysed for FOXP3 transcript. FOXP3 transcript was increased after *in vitro* challenge with gliadin. Foxp3 mRNA expression was normalized to the housekeeping gene GAPDH. The RNA was extracted from each patient's samples treated *in vitro* with medium alone or with gliadin. The experiment was repeated 4 times for each patient and the data reported in this figure were reproduced in the 4 separate experiments. One out of 4 independent experiments is shown.

4.2.3 Superantigen Staphylococcus Enterotoxin B up-regulates FOXP3 both in CD and in control mucosa.

It is not clear whether FOXP3 is a specific marker for Tregs in humans because it has been observed that activated T cells can transiently express FOXP3 (Gavin et al., 2006). Therefore to further investigate whether the gliadin induced $FOXP3^+$ up-regulation observed merely in CD mucosa was the result of a regulatory process taking place in CD mucosa rather than resulting in activation, I have treated small intestinal biopsies from CD patients and from controls with the toxin Staphylococcus Enterotoxin B (SEB). Toxins are poisons produced by living organisms. SEB is classified as exotoxin, since it is excreted by an organism, in this case Staphylococcus aureus bacterium, a Gram positive coccus. SEB normally exerts its effect in the intestine and therefore is termed as enterotoxin. SEB is commonly referred as a bacterial "superantigen" (SAg) because it is an extremely potent stimulator of T cell activation and in my study I have used it as a potential surrogate stimulator of gastrointestinal inflammation. The characteristic of a superantigen that render it "super" is that it is recognized by T cells without being processed into peptides that are captured by MHC molecules and it is able to stimulate the proliferation of a large number of T cells. SAgs can bind independently to MHC class II molecules and to T cell receptor (TCR), away from the binding region of the TCR that is highly specific for a specific antigen (CDR region, complementary determining region) and to the outer surfaces of MHC class II, outside the peptide binding site. A SAg can stimulate 2-20% of all T cells whilst an antigen can induce the activation of 0,001-0,0001% of T cells. This causes a massive immune response that is not specific to any particular epitope of the SAg.

Celiac and control mucosal explants were therefore cultured *in vitro* with peptic–tryptic digest of gliadin and with SEB for 24h and the frequency of $FOXP3^+$ cells was estimated before and after *in vitro* stimulation. These results showed that the number of $FOXP3^+$ cells was increased in both CD (with mean values: 28.60 ± 3.655 N=5) **(Fig. 4.7.a and b)** and control (with mean values: 14.00 ± 1.000 N=3) **(Fig. 4.8.a and b)** mucosa cultured in vitro with SEB. Importantly the accumulation of $FOXP3^+$ cells in CD mucosa after SEB challenge *in vitro* was much higher than in control mucosa. These data could suggest that the immune response against antigens able to induce a strong T cell proliferation is much more pronounced and sensitive in CD mucosa. These results also suggest that FOXP3 in CD is activated and up-regulated even by the SAg SEB. These results show that the number of $FOXP3^+$ cells is not increased in control mucosa after culture with gliadin but it is significantly increased when mucosa was cultured with SEB. These unexpected findings are unexplained. One possibility might be that FOXP3 is not a unique marker for Treg because its up-regulation followed by activation by SEB occurs in both CD and controls. These results could also mean that in CD a mechanism of suppressive regulation driven by Treg cells is lacking and so these cells are not able to modulate the pathogenic T cells gliadin specific, leading to the mucosal inflammation.



Untreated Gliadin SEB

Figure 4.7.a FOXP3 expression is induced in Lamina propria nuclei of CD mucosa upon activation *in vitro* with gliadin and with SEB. Celiac mucosal explants were cultured in vitro with medium alone (untreated), with gliadin and with SEB for 24h. The expression of the transcription factor FOXP3 was visualized by immunofluorescence. Arrows show nuclear transcription factor FOXP3 expression. The number of $FOXP3^+$ cells is increased after culture with SEB. The experiment was repeated 5 times for each patient and the data reported in this figure were reproduced in the 5 separate experiments.
Original magnification X 200.

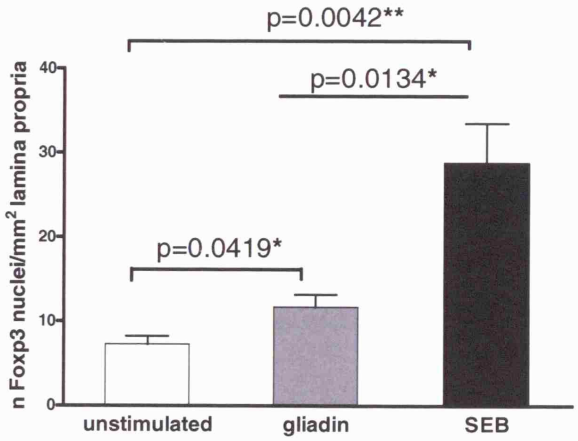


Figure 4.7.b - FOXP3 expression is induced in Lamina propria nuclei of CD mucosa upon activation *in vitro* with SEB. The quantification is based on the number of positive nuclei per area (mm^2) of lamina propria. FOXP3+ cells within the lamina propria were counted in 10 random hpf. Statistical significance was analysed by Student's *t*-test. Plots show mean values with SD bars.

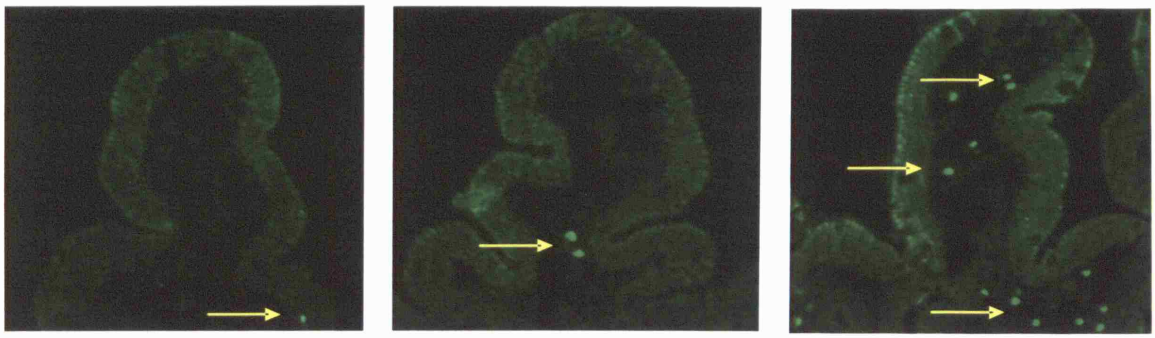


Figure 4.8.a FOXP3 expression is induced in Lamina propria nuclei of control mucosa upon activation *in vitro* with SEB. Control mucosal explants were cultured *in vitro* with medium alone (unstimulated), with gliadin and with SEB for 24h. The transcription factor FOXP3 staining was visualized by immunofluorescence. Arrows show nuclear transcription factor FOXP3 expression. The experiment was repeated 5 times for each control and the data reported in this figure were reproduced in the 5 separate experiments. Original magnification X 100.

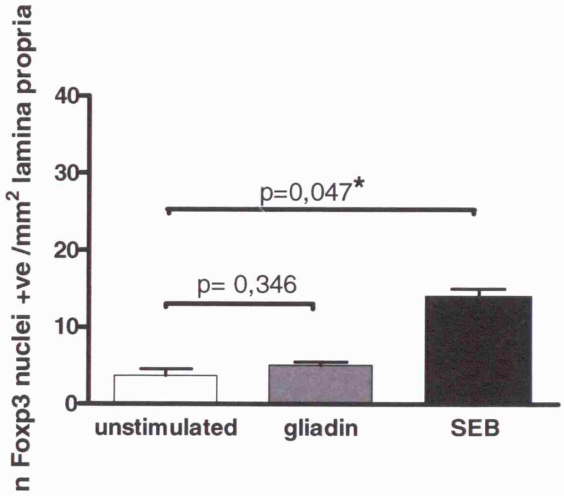


Figure 4.8.b - FOXP3 expression is induced in Lamina propria nuclei of control mucosa upon activation *in vitro* with SEB. The quantification is based on the number of $FOXP3^+$ positive nuclei per area (mm^2) of lamina propria. FOXP3+ cells within the lamina propria were counted in 10 random hpf. Statistical significance was analysed by Student's *t*-test. Plots show mean values with SD bars.

4.3 Discussion

The results of this chapter demonstrate for the first time that patients with CD have a significantly increased number of Lamina propria $FOXP3^+$ T cells compared with controls. Interestingly, in this study I observed also that gliadin is able to induce an up-regulation of the expression of both the Forkhead winged-helix transcription factor FOXP3 and its transcript in CD mucosa. I thus hypothesized that the increase of $FOXP3^+$ cells could represent a mechanism that could help broaden the Treg repertoire in CD mucosa and therefore down-regulate the gliadin-specific immune response. However, the larger increase in $FOXP3^+$ cells after SEB stimulation, in CD mucosa could suggest that these cells either might not be Tregs cells or might not be functioning at the mucosal level in CD patients. Further studies are needed to examine the interaction between SAg and Treg cells and their function at the mucosal level in patients with CD.

Since I was not able to get sufficient numbers of purified lamina propria $CD4^+CD25^+FOXP3^+$ T cells out of the mucosa in these studies because of the small size of the mucosal samples, and could not evaluate the function of these cells directly. Future studies are needed to clarify whether the observed increase of $FOXP3^+$ cells observed after *in vitro* challenge with gliadin renders these cells functional or not and also whether this enhancement of $FOXP3^+$ cells is due to an increase of activated T cells or increase of Treg cells without suppressive function in CD. Indeed, these results do not establish whether this induced increase of FOXP3 expression in these cells serves as a T cell intrinsic brake on the immune response against gliadin in CD.

In conclusion, these data demonstrate that gliadin-reactive $FOXP3^+$ cells are present in the intestinal mucosa of CD patients. I could not therefore hypothesize that the presence of

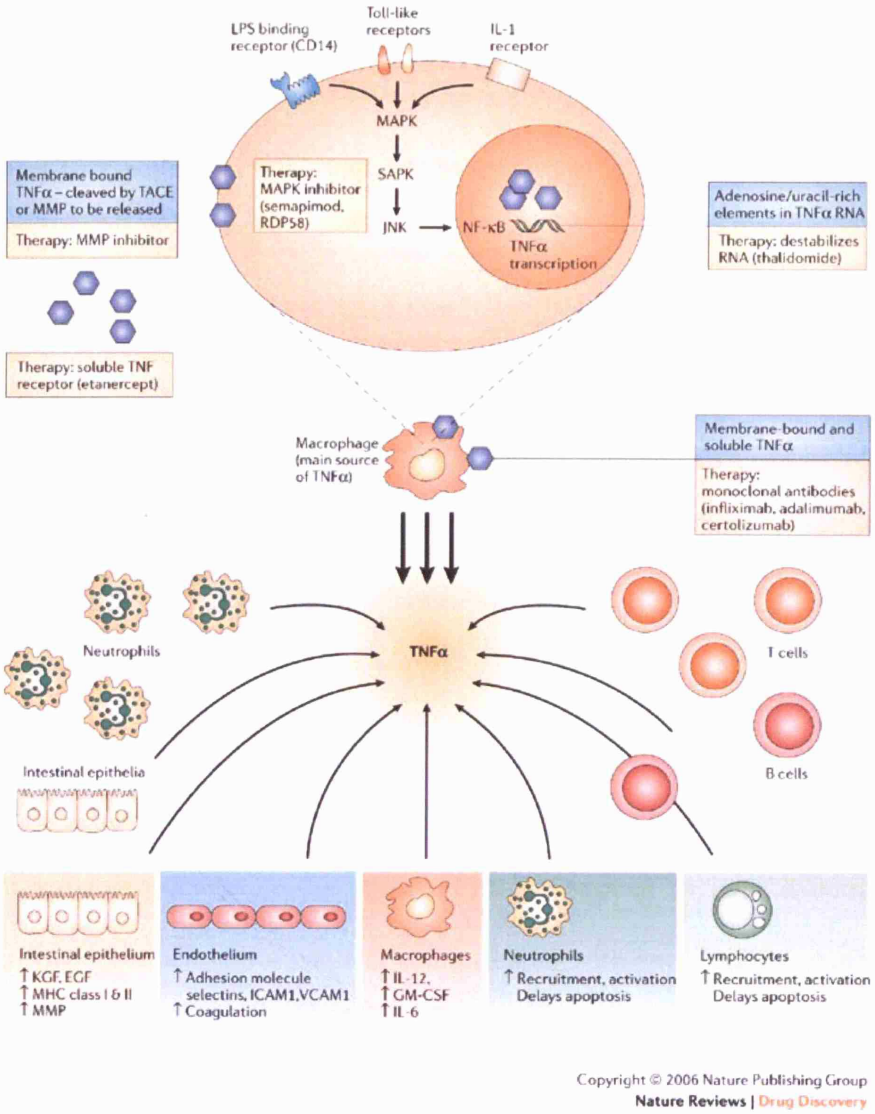
FOXP3$^+$ cells in CD patients could argue against a lack of function of FOXP3$^+$ cells as a cause of intestinal inflammation since the enhancement of the number of these cells in CD mucosa does not correspond in a suppressive immune response and in a protective effect towards gliadin or SAgs that are introduced into the gastrointestinal system.

Chapter 5 – Anti TNF-α therapy increases the frequency of FOXP3$^+$ Regulatory T Cells in Crohn's disease.

5.1 Introduction

The aetiology of chronic inflammatory diseases including Crohn's disease is not fully understood. The pathogenesis of Crohn`s is complex and multifactorial. A major current hypothesis establishes that the chronic inflammation in Crohn`s disease arises as a consequence of dysregulation of the adaptive immune system towards components of the intestinal flora, leading, in genetically predisposed individuals, to an immunological imbalance characterised by an excessive production of pro-inflammatory cytokines.

Crohn's disease exhibits an immunological response characterized by an exaggerated CD4 T helper cell type I phenotype. Thus, the pattern of cytokines expressed by lamina propria CD4 T cells is characterised by an increased production of inteleukin-12 (IL-12), interferon-gamma (IFN-γ, and Tumor Necrosis Factor-alpha (TNF-α). Despite redundancy among mediators of inflammation, a hierarchy of importance has emerged with TNF-α as a key effector and regulatory molecule in Th1 responses (Shanahan, 2000). TNF-α is believed to play a pivotal role in the initiation and in the amplification of the pathogenesis in Crohn's disease. TNF-α is a pleiotropic cytokine that is involved in the death of a variety of cell types (Locksley et al., 2001). TNF-α is considered the prototypical pro-inflammatory cytokine secreted by activated macrophages and monocytes and chronically activated T lymphocytes. Other cells known to produce TNF include B lymphocytes, natural killer (NK) cells, mast cells, intestinal mesenchymal and Paneth cells (Bischoff et al., 1999; Vassalli, 1992). In addition to these, TNF is also produced by intestinal epithelial cells in response to bacterial invasion (Jung et al., 1995).



Podolsky DK, *Nature Rev, Drug discovery*, 2006

Figure 5.1 The TNF-α pathway

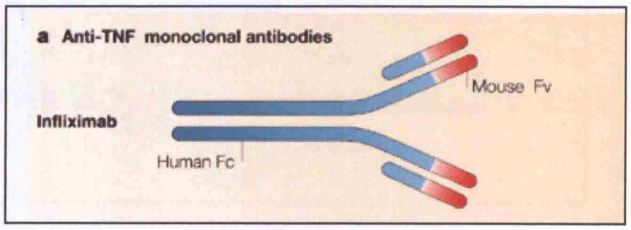
TNF-α is first produced as a 26-kilodalton trans-membrane form mTNF), which is cleaved into the secreted 17-kilodalton soluble (sTNF) form by TNF-α converting enzyme (TACE) (Black et al., 1997; Moss et al., 1997). Studies have shown that both the soluble and membrane portions of TNF-α are biologically functional (Bazzoni and Beutler, 1996). The

17-kilodalton form of TNF-α (sTNF) aggregates to tri-molecular complexes (trimers), which bind and activate their receptors (Papadakis and Targan, 2000). Furthermore, studies indicate that mTNF-a plays an important role in various immune responses (Monastra et al., 1996).

The TNF-induced responses are mediated through either one of its receptors, TNFR1 and TNFR2 that are functionally distinct (Papadakis and Targan, 2000). sTNF-α is predominantly produced by activated macrophages and lymphocytes (Vassalli, 1992) and preferentially binds to TNFR1, whereas mTNF-α preferentially binds to TNFR2 (Grell et al., 1995).

Several studies have detected increased TNF-α protein and mRNA levels in mucosal biopsies from patients with Crohn's disease (Present et al., 1999). The mechanism by which TNF-α regulates inflammation in the gut of a Crohn's patient is likely to be complex and multifactorial. To determine this mechanism, a series of *in vitro* experiments were performed using specimens from patients being treated with anti TNF-α. Among patients responsive to treatment with anti-TNF-α, there was sequential down-regulation of TNF-α and IFN-γ production in the mucosa. Th1 T cells were not eliminated; rather their function was reduced in inflamed mucosa to a level comparable with that seen in uninflamed mucosa (Plevy et al., 1997). This finding supports the notion that TNF-α augmentation of Th1 function in the mucosa is critical for disease pathogenesis. Therefore, TNF-α might be of particular importance in the mechanism leading to inflammation in Crohn's disease, as it regulates the production of other pro-inflammatory mediators; hence, the rationale for its use as a therapeutic target.

Several TNF-α neutralizing antibodies and fusion proteins have been reported to be clinically beneficial in chronic inflammatory diseases (Feldmann and Maini, 2001; Sandborn and Hanauer, 1999). In particular, Infliximab, a chimeric monoclonal antibody (three quarters human-constant region, human immunoglobulin IgG1, and one quarter mouse, variable region, Fv), is highly effective in the treatment of steroid-refractory Crohn's disease, as it induces clinical remission (Targan et al., 1997).

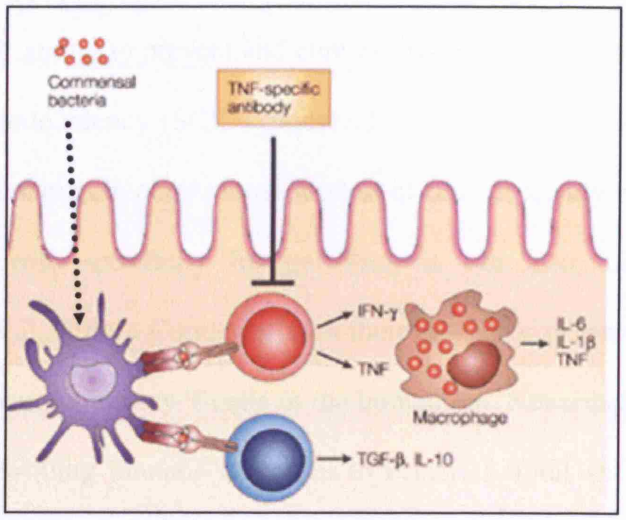


Feldmann M, *Nat Rev Immunol*, 2002

Figure 5.2 Anti TNF-α antibody Infliximab

Infliximab is able to neutralize both forms of TNF-α, by either binding the membrane-bound TNF-α (mTNF) and/or by blocking the soluble cytokine (sTNF) (Papadakis and Targan, 2000) and is a highly effective treatment in Crohn's disease. However other anti TNF strategies which block TNF signalling (e.g. Etarnecept) that is effective in the improvement of RA, are ineffective in Crohn`s. An explanation to this could be that Etarnecept binds just the sTNF and not the mTNF as Infliximab does. Infliximab treatment achieves positive effects in 60% of Crohn's patients; however, the exact mechanism is unclear (Van den Brande et al., 2003). The benefit of Infliximab might be due to the induction of apoptosis and/or promotion of lysis of inflammatory cells, both monocytes and lamina propria T lymphocytes, bearing TNF-α on the cell surface. The pathway involved in

immune cell apoptosis induced by Infliximab seems to require Caspase-3 activation and does not involve CD95/CD95L (Fas/FasLigand) dependent mechanism (Di et al., 2004).



Bouma G, Strober W, *Nature Rev Immunol*, 2003

Figure 5.3 Points of Infliximab therapeutic attack.

Recent studies in Rheumatoid arthritis (RA) have revealed a novel and potentially important immunoregulatory action of TNF-α suggesting that this cytokine is able to down regulate CD4+CD25+ T reg function (Valencia et al., 2006). This TNF-mediated inhibition of suppressive function is related to a decrease in FOXP3 mRNA and protein expression by Tregs and is mediated through TNFR2. They demonstrate that human Tregs express TNFR2 and that the percentage of Tregs that express TNFR2 can be enhanced by exposure to TNF. Hence this cytokine has been found to be important in the homeostasis of the T reg subsets. These findings contrast with another recent study in mice were it has been shown that TNF up-regulates Foxp3 expression and immunosuppressive activity of Tregs) (Chen

et al., 2007). This discrepancy is likely due to the species differences of Treg response to TNF-α.

Treg cells may also have an important role in tolerance towards the microbial flora, as suggested by their ability to prevent and cure colitis in mouse models of IBD in the severe combined immunodeficiency (SCID) mouse. Treg cells have been shown to significantly control gut inflammation in other mouse models of IBD. Recently it has been demonstrated that Treg cells with specificity for gut bacteria can also inhibit colitis in animal models(Powrie et al., 1994). Compared with their characterization in the mouse, relatively little is known about regulatory T cells in the human gut. Nevertheless, the possible role of Treg cells in controlling immune responses to bacterial floral antigens in both mice and humans is a question that needs further investigation.

There is considerable data suggesting that the function of Tregs is governed by the effect of a transcriptional repressor, FOXP3. In animals and humans lacking FOXP3 expression, Treg function is absent (Bennett et al., 2001; Fontenot et al., 2003; Khattri et al., 2003) whereas over-expression of FOXP3 by *in vitro* transfection induces T cells both to become anergic and to exert a complete (Yagi et al., 2004) or partial suppressive activity (Allan et al., 2005). Thus expression of FOXP3 appears to play a necessary role in governing Tregs action. Reduced function of Tregs may be involved in the breakdown of immunological self-tolerance in patients with multiple sclerosis (Viglietta et al., 2004) and in active rheumatoid arthritis (Ehrenstein et al., 2004), thus I investigated whether defective properties of Tregs play a role in Crohn's disease and also whether the beneficial effects of TNF blockade by in vivo treatment of Crohn's patients could involve the restoration of

immune homeostasis by permitting the full expression of $CD4^+CD25^+FOXP3^+$ T regs function.

5.2 Results

5.2.1 $FOXP3^+$ cell numbers are reduced in the mucosa of Crohn's patients with active disease compared with controls.

To study the Tregs in Crohn`s disease and to try to understand the role, if any, of these cells in the pathogenesis of the disease, I have assessed whether Treg frequency was different between Crohn's and control mucosa by looking at the expression of $FOXP3^+$ cells.

At the beginning of my study I had a look at the Tregs as a percentage of CD4+CD25+FOXP+ cells by isolating these cells from the intestinal lamina propria and analyse the data by flow cytometry.

The data obtained showed that FOXP3 was expressed in all the CD4+CD25+ population isolated. Unfortunately, due to the small size of the samples the number of T reg cells obtained was not sufficient to get enough conclusions and from that the decision to use FOXP3 as sole marker to identify Tregs in the mucosa.

Colonic mucosal biopsies from active Crohn`s and from controls (children being investigated for constipation in whom inflammation was absent in routine laboratory histology) were compared. In these experiments the superantigen Staphylococcus Enterotoxin B (SEB), a potent stimulator of T cells, was used *in vitro* as a potential surrogate stimulator of gastrointestinal (GI) inflammation. The frequency of $FOXP3^+$ cells was estimated before and after in vitro stimulation with SEB. Biopsy samples were cultured *in vitro* for 24h in the presence of medium alone and SEB. To define T reg cells in the

mucosal samples, FOXP3 expression in nuclei of Lamina Propria cells was determined by immunoperoxidase staining using specific monoclonal antibodies. The results showed that the number of $FOXP3^+$ cells was reduced in mucosa of Crohn's patients with active disease (**Fig 5.5** (N=4)) compared with controls (**Fig 5.4** (N=3)). These results supported the finding that the number of T reg cells is lower in Crohn`s mucosa compared with controls. The number of $FOXP3^+$ cells increased after *in vitro* challenge with SEB in both Crohn`s (N=4) and in control mucosa (N=3) (**Fig 5.5 and Fig 5.4**).

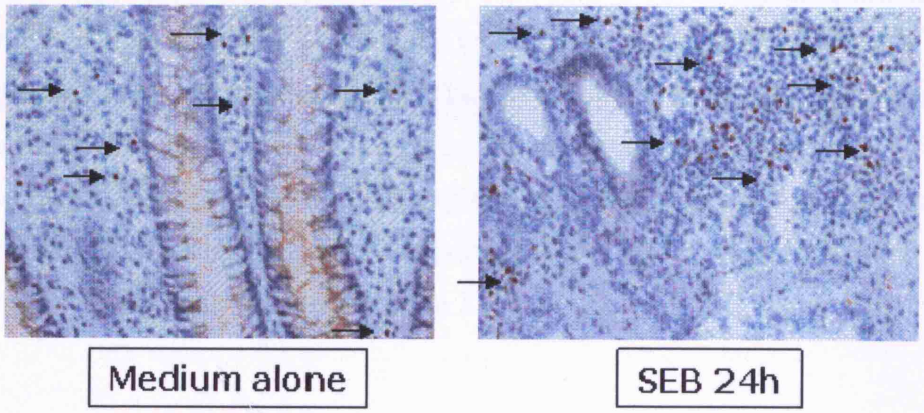


Figure 5.4 FOXP3 cells in control mucosa cultured in vitro with medium alone and SEB for 24h. Colon biopsies from normal controls were cultured *in vitro* fro 24h with medium alone or with SEB. The transcription factor FOXP3 staining was visualized by immunoperoxidase. Nuclei were counterstained with Haematoxylin. Arrows show nuclear transcription factor FOXP3 expression. The experiment was repeated 5 times for each control and the data reported in this figure were reproduced in all 5 separate experiments. Original magnification X 100.

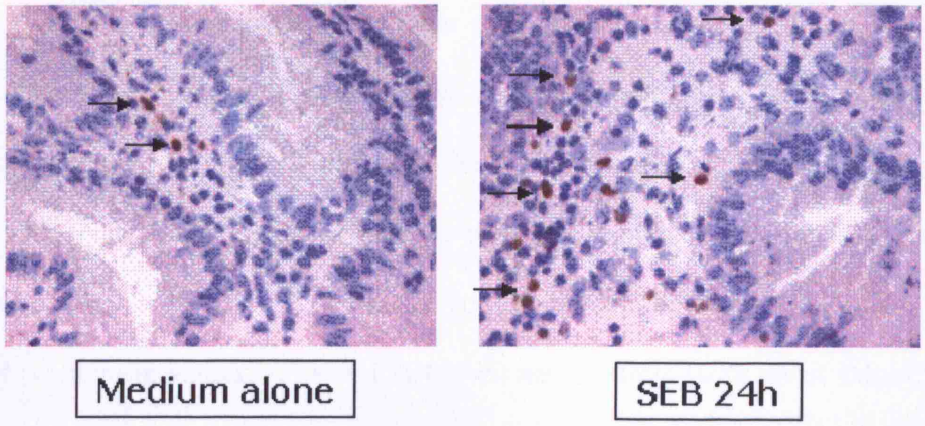


Figure 5.5 FOXP3 cells in Crohn's mucosa cultured in vitro with medium alone and SEB for 24h. Colon biopsies from patients with active disease were cultured *in vitro* fro 24h with medium alone or with SEB. The transcription factor FOXP3 staining was visualized by immunoperoxidase. Nuclei were counterstained with Haematoxylin. Arrows show nuclear transcription factor FOXP3 expression. The experiment was repeated 5 times for each Crohn's patients and the data reported in this figure were reproduced in all 5 separate experiments. Original magnification X 200.

5.2.2 FOXP3 increases in mucosa of Crohn`s patients treated *in vivo* with Infliximab compared with mucosa of untreated Crohn`s patients and controls.

It is generally accepted that the pro-inflammatory cytokine TNF-α is a key cytokine in the pathogenesis of Crohn's disease. Hence, I set out to investigate whether the defect in the number of regulatory T cells that I observed in the colonic mucosa of patients with active Crohn's disease was due to TNF-α. To determine this, I have investigated whether anti-TNF-α therapy, that has been observed to markedly ameliorate disease activity in many patients, resulted in changes in this T cell population. For this part of the study, biopsies from Crohn`s patients receiving treatment with Infliximab, Crohn`s not receiving Infliximab and controls were analysed to investigate whether there was any significant

difference either in the frequency of number of nuclei expressing the transcription factor FOXP3 or in the expression of its transcript. Biopsy samples were also cultured *in vitro* with medium alone and with SEB for 24h. Nuclear expression of the transcription factor FOXP3 was significantly higher in mucosa of Crohn`s patients treated with Infliximab (with mean values: 24.00 ± 3.071 N=7) compared with mucosa of Crohn`s patients untreated (with mean values: 7.000 ± 1.304 N=5) and controls (with mean values: 11.75 ± 1.377 N=4) **(Fig 5.7 and Fig 5.8).** In mucosal biopsies collected from patients treated *in vivo* with Infliximab we observed that the number of positive FOXP3 nuclei was moderately invariant between samples cultured in medium alone compared with samples challenged *in vitro* with SEB (**Fig 5.6**).

In these experiments RT-PCR was also performed to quantify the FOXP3 transcript in mucosa from Crohn`s patients treated or not with Infliximab. Results with RT-PCR also showed an up-regulation of FOXP3 transcripts in colonic mucosa from Crohn`s patients treated with Infliximab compared with mucosa from untreated patients (**Fig 5.9**). Tregs from patients with Crohn`s disease expressed reduced levels of FOXP3 mRNA and protein. Treatment with anti-TNF antibody (Infliximab) *in vivo* increased FOXP3 mRNA and protein expression by Tregs.

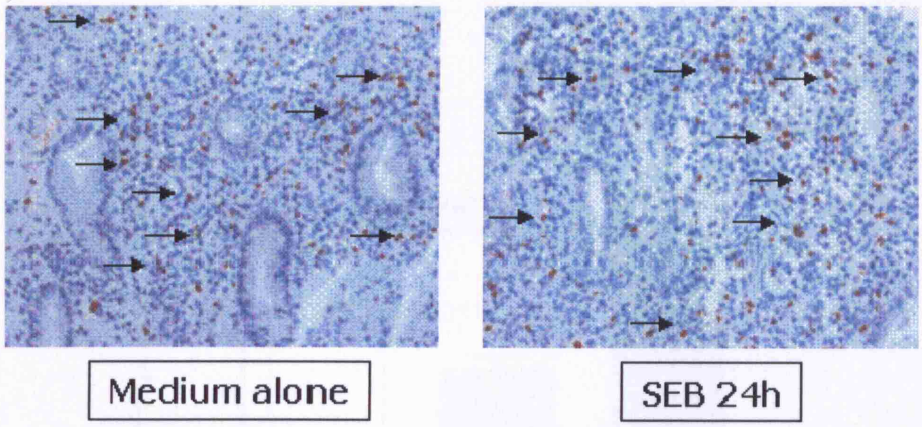


Figure 5.6 FOXP3 cells in Crohn's mucosa from patients treated with Infliximab and cultured in vitro with medium alone and SEB for 24h. Colon biopsies from Crohn`s patients treated *in vivo* with Infliximab were cultured *in vitro* fro 24h with medium alone or with SEB. FOXP3 staining was visualized by immunoperoxidase. Nuclei were counterstained with Hematoxylin. Arrows show nuclear transcription factor FOXP3 expression. The experiment was repeated 5 times for each Crohn's patient treated with Infliximab and the data reported in this figure were reproduced in all 5 separate experiments. Original magnification X 100.

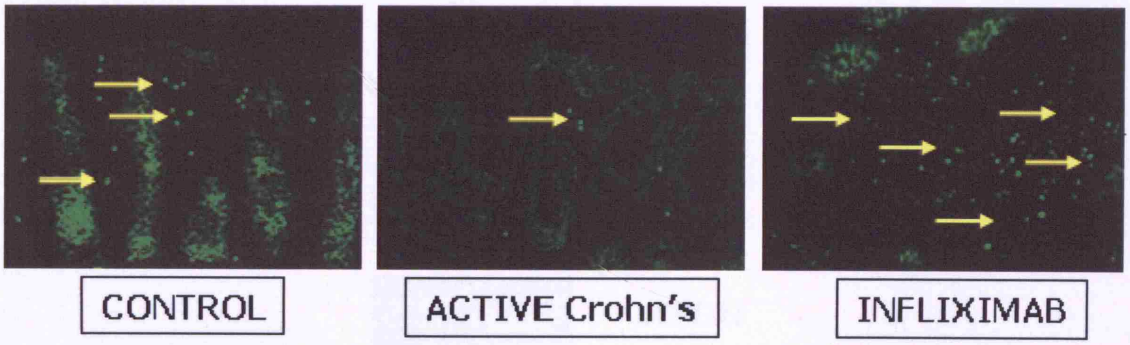


Figure 5.7 Expression of FOXP3 in colon mucosa lamina propria of controls, active and Infliximab treated Crohn's patients. Colon biopsies from normal controls, Crohn`s patients with active disease or treated *in vivo* with Infliximab were analysed. FOXP3 staining (green) was visualized by immunofluorescence and analyzed using conventional fluorescence microscopy. Arrows show nuclear transcription factor FOXP3 expression. The experiment was repeated 5 times for each control and each patient and the data reported in this figure were reproduced in all 5 separate experiments. Original magnification X 100.

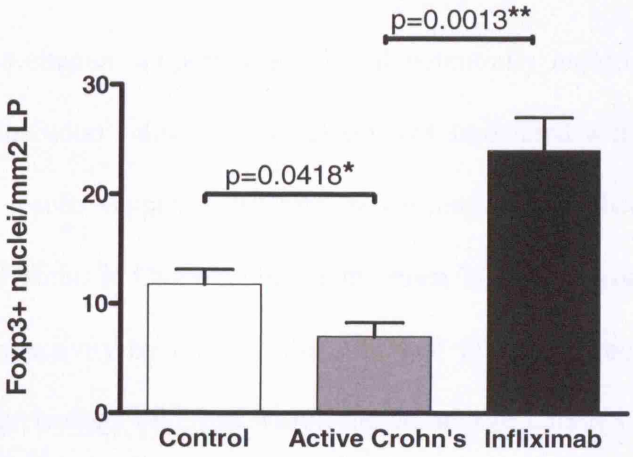


Figure 5.8 FOXP3$^+$ nuclei expression in Lamina propria of colon mucosa from controls, active and Infliximab treated Crohn's patients. The quantification is based on the approximate number of positive nuclei per area of lamina propria. The quantification is based on the number of FOXP3$^+$ positive nuclei per area (mm^2) of lamina propria. FOXP3+ cells within the lamina propria were counted in 10 random hpf. Statistical significance was analysed by Student's *t*-test. Plots show mean values with SD bars.

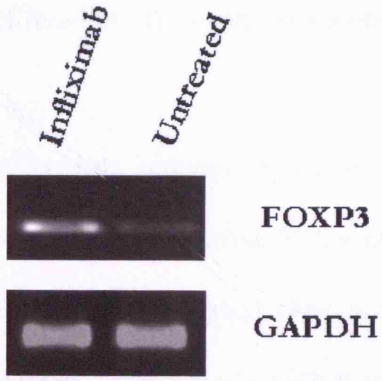


Figure 5.9 FOXP3 transcript was increased in the mucosa of Crohn's patients treated *in vivo* with Infliximab. Colonic biopsy specimens from Crohn's patients treated or not in vivo with Infliximab were analysed. mRNA isolated from intestinal biopsies of all the patients was subjected to RT-PCR and analysed for FOXP3 transcript. FOXP3 transcript was increased in the mucosa of Crohn's patients treated in vivo with Infliximab. Foxp3 mRNA expression was normalized to the housekeeping gene GAPDH. One out of 4 independent experiments is shown.

5.3 Discussion

The results of this chapter support a novel and potentially important immunoregulatory action of TNF-α in Crohn's disease. This effect was associated with the down-modulation of FOXP3. These results suggest a potential novel interaction between the innate and the adaptive immune systems in Crohn's disease in which TNF-α, a product of the former, can promote immune reactivity by limiting the action of $FOXP3^+$ Tregs. The results provide new insight into the biology of Tregs within the context of Crohn's disease. During initial tissue invasion by colonic microrganisms, exuberant TNF production may limit the activity of Tregs by binding to the TNFR2, and promote induction of immune reactivity and the effector phase of lymphocyte responses. It is conceivable that TNF-α has a direct effect on regulatory T cells viability, such as the induction of apoptosis (Sarin et al., 1995) through the binding mTNF-α with TNFR2. So in Crohn's disease the loss of mucosal homeostasis with the increase of T cell proliferation (Th1) and the apoptosis of Tregs is due to the effect of TNF-α.

TNF-α neutralization by Infliximab induces apoptosis of T effector cells via the mitochondrial pathway with production of Caspase-3. Apoptosis in Tregs is induced via the activation of the CD95/CD95L (Fas/FasL) pathway (Fritzsching et al., 2005).

As TNF-α is blocked by Infliximab, a decrease in mTNF-α may result in less activation of Tregs through TNFR2 and in no Treg apoptosis which would explain the increased number of $FOXP3^+$ Tregs after TNF-α neutralization in the results presented in this chapter.

With the enhancement of Tregs function immunological tolerance is restored and therefore limiting immune reactivity to tissue antigens revealed during inflammation.

Another explanation of the increase of $FOXP3^+$ cells could be due to the fact that Infliximab induces apoptosis of pathogenic T cells through the mitochondrial pathway, pathway that Tregs are not sensitive to. This might explain why treatment of patients with Infliximab does not induce apoptosis of Tregs. In this way, TNF-α may play an important instructive role in controlling adaptive immunity.

The manipulation of TNF-α signalling in Tregs may result in novel therapeutic approaches to augment the limited and/or inadequate function of these $FOXP3^+$ Tregs in Crohn's disease.

Treatment *in vivo* with anti TNF-α antibody resulted in a significant clinical benefit although it could not be proved whether such change was an epiphenomenon or a primary therapeutic event.

Further studies are required to elucidate the mechanisms that underlie these observations in patients with Crohn's disease, and to relate the clinical and immunological responses in patients receiving anti-TNF therapy. Restoration of the function of Treg cells or transfer of fully competent Treg cells could be a useful therapeutic tool in the treatment of Crohn's disease.

Chapter 6 – Treatment of children with fulminating ulcerative colitis (UC) with Basiliximab decreases the number of FOXP3+ Treg cells and results in clinical benefits.

6.1 Introduction

The aetiology of Ulcerative Colitis (UC) is largely unknown, although it is clear that a combination of hitherto unidentified genetic and environmental factors results in a chronic inflammatory reaction (Bouma and Strober, 2003). In UC the inflammation is driven by activated T-lymphocytes, which have a T helper 2 cytokine profile. These T-cells recruit neutrophils and monocytic phagocytes to the colonic mucosa, resulting in hemorrhagic ulcerations. In recent years, new biological therapies aimed specifically at eliminating inflammatory mediators have been introduced in IBD therapy. As already mentioned in chapter 5 and in the introduction, anti-tumour necrosis factor-α (TNF-α) agents are now being used in daily practice to treat refractory Crohn's disease. Biological therapies for UC have not yet developed to the same extent as in Crohn's disease. The standard treatment for UC has been anti-inflammatory agents such as sulfalasine and mesalazine (5-aminosalicylic acid (5-ASA)) in combination with corticosteroids for severe inflammation (Bitton et al., 2001). Nevertheless severe steroid refractory cases of UC can be managed by either medical immunosuppression or by total colectomy. Medical immunosuppressive therapy usually consists of a therapy with cyclosporine-A (CsA). Cyclosporine A, a fungal metabolite, down-regulates lymphocytes activation and proliferation by selectively inhibiting the transcription of IL-2 in activated T-cells through the inhibition of the calcineurin pathway. CsA binds to and activates the phosphatase calcineurin responsible for

activating nuclear factor of activated T-cells (NFAT), which is a transcription factor for IL-2 synthesis. IL-2 interacts with specific receptors on the T-lymphocyte membrane to induce a clonal expansion and proliferation of T-effector cells. In some refractory cases, colectomy can be avoided using this treatment strategy. However, the use of both corticosteroids and cyclosporine is associated with important side effects. The leading side effects of cyclosporine therapy include renal toxicity, hypertension and neuropathy. Nevertheless, the efficacy of medical immunosuppression with cyclosporine in the treatment of moderate to severe UC has been established (D'Haens et al., 2001; Lichtiger et al., 1994).

Recently a number of newer immunosuppressant therapies have been explored, with particular targeting of the pro-inflammatory cytokine IL-2. IL-2, which is one of the first cytokines identified, acts at the heart of the immune response (Smith, 1988). IL-2 and its alpha receptor, IL-2Rα, are expressed by T cells after the activation of T cell receptors by peptide-major histocompatibility complexes. The subsequent autocrine interaction of IL-2 with its receptors leads to the stimulation of signal transduction pathways resulting in T cell, B cell, and natural killer (NK) cell proliferation and clonal expansion (Nelson and Willerford, 1998). IL-2 has paradoxical functions in T cell homeostasis, acting as a potent T cell growth factor during the initiation of immune responses and having a crucial function in the termination of T cell responses and maintenance of self tolerance (Malek and Bayer, 2004). The latter function has been proposed to be due to a requirement for IL-2 signalling for the development and function of regulatory T cells (Treg), the naturally occurring population of $CD4^+$ T cells that are vital in the control of autoimmune and inflammatory responses (Sakaguchi, 2004). Studies have clarified the importance of IL-2 signalling in $CD4^+CD25^+FOXP3^+$ Treg cell homeostasis, concluding that although IL-2 signalling is not

required for their development in the thymus, it is critical for maintaining T cells in the peripheral T cell pool (D'Cruz and Klein, 2005; Fontenot et al., 2005). The pleiotropic biological activities of IL-2 are mediated either through a high affinity hetero-trimeric receptor complex consisting of the IL-2Rα chain (CD25); the IL-2Rβ chain (CD122); the common gamma chain (CD132, or γc), or through an intermediate affinity hetero-dimeric receptor complex composed solely of CD122 and CD132. Whereas the CD25 receptor is exclusively used for IL-2 binding, CD122 is also part of the IL-15 receptor and CD132 is used by IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Kovanen and Leonard, 2004). These cell surface receptors form a complex that signals through the intracellular activation of the Janus tyrosine kinase 3 (Jak3) and the signal transducer and activator of transcription 5 (STAT5) (Minami et al., 1993).

The IL-2Rα chain, originally identified as the Tac antigen (CD25) (Leonard et al., 1982; Leonard et al., 1984) is absent or low on resting cells but transiently increases following diverse stimuli (Ascherman, 1997; Sivori, 2004), while CD122 and CD132 expression is constitutive (Taniguchi and Minami 1993).

CD25 is a target for therapeutic modulation because it is not expressed on resting T and B cells but it is continuously expressed by pathologic T cells that turn on genes that help T cells to proliferate and differentiate into effector T cells and also by T reg to balance immune homeostasis.

Notably, the transcription factor FOXP3, identified as crucial for the development and function of the naturally occurring $CD4^+CD25^+$ Tregs, inhibits the transcription of the gene encoding IL-2 and up-regulates the expression of CD25. The resulting inability of $FOXP3^+$ Treg cells to produce IL-2 appears to make them highly dependent on exogenous IL-2 for

survival. Accordingly, mice genetically deficient in IL-2, CD25, or CD122 and humans with genetic deficiency of CD25 have both reduced numbers and impaired function of $FOXP3^+$ Treg cells and succumb to severe autoimmune inflammatory diseases (Sakaguchi, 2005; Sakaguchi and Powrie, 2007). Furthermore, neutralization of circulating IL-2 by anti-IL-2 antibody treatment specifically reduces the number of $CD4^+CD25^+$ T cells, producing organ-specific autoimmune diseases as observed after Tregs depletion in mice (Setoguchi et al., 2005).

In humans antagonistic monoclonal antibodies targeting the proinflammatory cytokine IL-2 and neutralizing the binding capacity of the high affinity IL-2 receptor on antigen-exposed T-lymphocytes have been developed. Basiliximab (Simulect) is a chimeric IgG1 monoclonal antibody directed against CD25 and it therefore competes with IL-2 in binding to the high-affinity receptor has been observed effective in preventing the rejection of organ transplants (Amlot et al., 1995) .

Anti CD25 therapies are currently used during intestinal transplantation, and for the treatment of severe intestinal graft versus host disease following allogenic haematopoietic stem cell transplantation (Calmus et al., 2002; Lawen et al., 2003; Martin et al., 2004). Previous studies have suggested that anti CD25 therapy is beneficial in steroid resistant UC (Creed et al., 2003; Schwarzer et al., 2006). The underlying hypothesis for the development of this drug has been that blocking of the IL-2 receptor (CD25) may prevent the proliferation and differentiation of the pathogenic effector T cells.

Several lines of evidence suggest that the generation and peripheral homeostasis of $CD4^+CD25^+$ T reg cells are intimately entwined with the action of IL-2. Of note, CD4+CD25+ cells produce negligible IL-2 of their own (Thornton and Shevach, 1998): IL-

2 must be provided by another T cell subset. In murine models of inflammatory bowel disease, the proposed role of IL-2 in the pathogenesis of colitis has been uncertain. Knockout mice with a targeted disruption of IL-2 or IL-2Rα develop inflammatory bowel disease(Poussier et al., 2000; Sadlack et al., 1993).

However, studies in the field of regulatory T cells (Tregs) have shown that CD25 represents a crucial marker of $FOXP3^+$ T cells with suppressor function (Bluestone and Abbas, 2003; Sakaguchi, 2004; Shevach, 2002).

Recent studies have revealed a functional role for CD25 expression on $CD4^+$ T reg cells such that interruption of the IL-2/IL-2R signalling pathway blocks Treg effector function potentially via alteration in the expression of the glucocorticoid-induced TNFR-family gene (GITR) (Kohm et al., 2005; Kohm et al., 2005) (Thornton et al., 2004). Accordingly, a number of groups have targeted CD25 as a mechanism of depleting T reg cells and studying resultant effects on T cell activation, trafficking, and/or function. It is widely believed that injection of anti CD25 mAb results in the rapid and efficient depletion of $CD4^+CD25^+$ T reg cells, as determined by secondary immunostaining with mAb anti CD25 (McHugh and Shevach, 2002) (Onizuka et al., 1999) In recent studies it has been reported that in vivo injection of anti CD25 mAb in mice resulted in the functional inactivation but failed to physically deplete $CD4^+CD25^+$ T reg cells resulting in an exacerbation of acute clinical experimental autoimmune encephalomyelitis (EAE) (Kohm et al., 2006). Supporting this, mice receiving anti-CD25 mAb treatment displayed significantly lower numbers of $CD4^+CD25^+$ T cells but no change in the number of $CD4^+Foxp3^+$ Treg cells. Other studies in mice also revealed that transient depletion of $CD4^+CD25^+$ Treg in vivo

using anti CD25 resulted in severe spontaneous autoimmune thyroiditis (SAT) (Yu et al., 2006).

The same might be expected in UC but recent clinical studies by our group and by others have shown that the use of the anti CD25 antibody Basiliximab was efficacious in the improvement of fulminating ulcerative colitis (Creed et al., 2006) (Schwarzer et al., 2006) The patients used for this study were poorly responsive to combined therapy with steroid and CsA and were treated *in vivo* with anti CD25 antibody Basiliximab. After this immunosuppressant treatment clinical disease activity scores were normalised within 72 h of Basiliximab administration and colonic histology provided also evidence of mucosal healing within 10-14 days (Schwarzer et al., 2006). All four patients used in this study were at high risk of colonic perforation and consequent colectomy. All of them demonstrate a rapid improvement in clinical status following a single infusion of anti-CD25. None of the patients required colectomy with 60 days and all returned home on normal diet. This small cohort of children with UC demonstrate the proof of principal aggressive anti IL-2 therapy is able to bring about a rapid improvement in clinical colitis scoring in association with resolution of the severe mucosal inflammatory process in UC.

Previous studies have shown that $CD4^+CD25^+FOXP3^+$ Treg cells are expanded in inflamed and uninflamed colon in UC compared with control (Yu et al., 2007). The changes of these cells number in UC mucosa lead me to think that their function is impaired as well and this could therefore be one of the causes of the onset of the disease. Alternatively, Treg cells may be functionally normal in UC and just quantitatively increased during the development of the disease.

In this chapter I have investigated whether the beneficial effect of anti-CD25 blockade by *in vivo* treatment of UC patients could have any effect in the blockade or depletion of $FOXP3^+$ T regs cells.

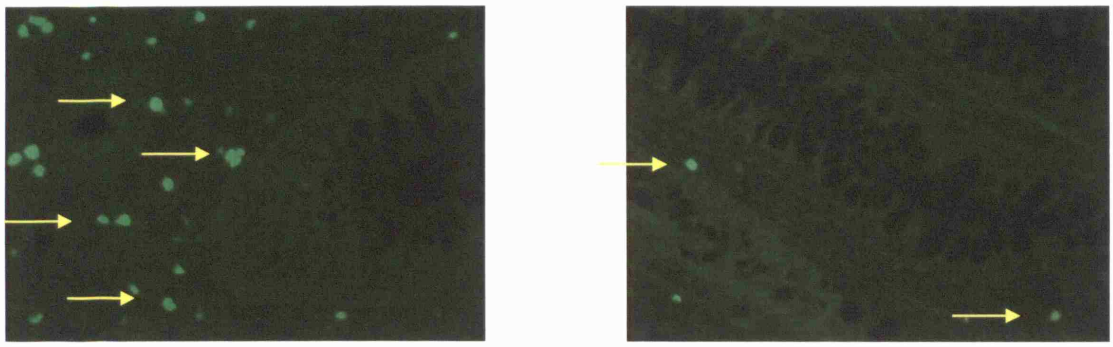
6.2 Results

6.2.1 FOXP3 decreases after the treatment *in vivo* with anti-CD25 antibody in colonic lamina propria cells of UC patients.

To study the T regs in UC and to try to understand the role, if any, of these cells in the pathogenesis of the disease, I have assessed whether Tregs frequency, was different between UC patients before and after treatment in vivo with anti-CD25 (Basiliximab), by looking at the expression of $FOXP3^+$ cells. Colonic mucosal biopsies from active UC before and after 10-14 days of anti CD25 therapy were compared to investigate whether there was any significant difference either in the frequency of number of nuclei expressing the transcription factor FOXP3.

Nuclear expression of the transcription factor FOXP3 (**Fig 6.1a**) was significantly lower in mucosa of UC patients treated with Basiliximab (with mean values : 19.25 ± 0.4787 N=4) compared with mucosa of the same patient before the treatment (with mean values: 4.750 ± 0.4787 N=4) **($p<0.0001$) (Fig 6.1.b).**

These results show that the anti CD25 antibody was physically depleting $FOXP3^+$ cells and demonstrate that the clinical improvement in UC was associated with a specific inhibition of IL-2 receptor signal transduction.



Before anti CD25 After anti CD25

Figure 6.1.a FOXP3 expression is decreased in Lamina propria nuclei of UC mucosa after anti CD25 therapy (Basiliximab). Colon biopsies from UC patients before and after 10-14 days of Basiliximab treatment were analysed. The expression of the transcription factor FOXP3 was visualized by immunofluorescence. Arrows show nuclear transcription factor FOXP3 expression. The number of $FOXP3^{+}$ cells is decreased after anti-CD25 therapy. Original magnification X 200.

The experiment was repeated 4 times for each patient and the data reported in this figure were reproduced in the 4 separate experiments.

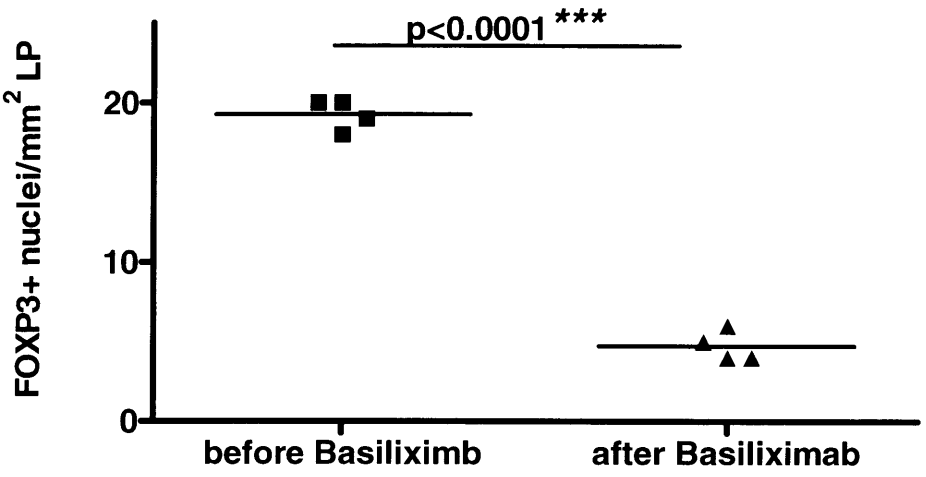


Figure 6.1.b FOXP3 expression decreases significantly in UC mucosa after treatment *in vivo* with Basiliximab. The quantification is based on the number of FOXP3$^+$ positive nuclei per area (mm^2) of lamina propria. FOXP3+ cells within the lamina propria were counted in 10 random hpf. Statistical significance was analysed by Student's *t*-test. Plots show mean values with SD bars.

6.3 Discussion

The aetiology of UC is not completely understood, current therapies target the immune response generating autoimmunity and inflammatory processes within the intestine.

As it has been observed that either targeting the IL-2 pathway or the IL-2 receptor CD25 is therapeutically useful in the management of the disease, I investigated whether the use of the immunotherapy drug targeting IL-2 receptor was related to any variance of FOXP3+ Treg cells.

These data challenge the hypothesis that IL-2 receptor activation in established human UC has an anti-inflammatory as already showed in clinical trials.

Therapy with anti CD25 might have two opposing effects targeting the pro-inflammatory effect of IL-2 on the effector T cells, but also depleting CD25 regulatory T cells.

These results are not in line with previous results observed in other diseases where the depletion of Tregs induced or worsened the autoimmune disease. With these results, if translated into clinical benefits observed in the disease progression I would hypothesize that other mechanisms may be responsible for keeping the inflammatory reaction in the intestine of UC at bay.

Further studies are needed to be performed to characterize the nature of these cells whether they are effector T or T reg cells and also whether FOXP3 expression in UC is just transient as a characteristic of T effector cells or is stable like in Tregs.

Chapter 7 – General Discussion

7.1 Role of Rho-A in the innate immune response to gluten in CD.

The epithelial surface of the intestinal mucosa, as a first line of host defense, has a crucial role in the innate immune response towards food antigens such as gluten peptides that are recognized as non-self antigens in CD and able to induce the pathogenesis of CD. At the centre of this pathogenic cascade is the recognition by resident CD4+ T cells of well defined fragments of gliadins, typically α-gliadin, as well as other gluten components such as glutenins. A series of studies pioneered by our group (Maiuri et al., 2003) have also indicated that gluten, in particular a small fragment of the α-gliadin p31-43, induces a series of non adaptive mucosal modifications at the level of the epithelium as well as on resident myeloid cells, such as dendritic cells (Maiuri et al., 2003). The modalities of action of this second pathogenic pathway are far from clear and to date we have few clues as to the molecular mechanisms involved.

The morphological changes observed in the mucosal enterocytes also strongly suggest that the cytoskeleton may be involved in the mechanism of epithelial cell damage observed in CD. The innate immune response to gluten, (i.e. p31-43 or 31-49) has been reported by several groups to be characterized by specific morphological changes such as induction of actin reorganization with induction of stress fibers. Since regulation of most actin-dependent processes has been demonstrated to be mediated by the Rho family GTPases signaling pathway, in this thesis I have investigated whether Rho was involved in these morphological changes and induced in the epithelial cells of CD mucosa.

My results indicate that the gliadin peptide induced modifications; in particular that tyrosine phosphorylation and actin reorganization were highly sensitive to Rho-A

neutralization stressing the role of this signal transduction pathway in immune innate activation by gliadin. I however observed that apoptosis induction was not significantly controlled by Rho-A inhibition but specifically via inhibition of the down-stream effector ROCK-I. ROCK-I can be activated also by alternative pathways leading to cell death such as the Caspase-3 mediated pathway.

My results thus support a central role of Rho-A in gliadin induced modification but also suggest that alternative pathways are activated, and the significance of the results will be discussed in the context of CD.

7.2 FOXP3$^+$ Regulatory T cells in the immune response to gluten in CD.

In CD the self-tolerance towards gliadin is broken. An activation of the pathogenic T cells in the lamina propria leads to an uncontrolled immune response that induces inflammation and tissue damage in the small intestine. The role of FOXP3$^+$ cells as a regulatory factor able to eventually control the mucosal tolerance has never been investigated in CD.

The data shown in this thesis show that FOXP3$^+$ cells are increased in CD mucosa compared with controls. These results lead me to predict that a process of immuno-regulation is taking place in the inflamed CD mucosa. I also observed that FOXP3$^+$ cells were up-regulated after challenge in vitro with gliadin. These data allow me to hypothesize that FOXP3$^+$ cells are antigen specific in CD. When I cultured *in vitro* mucosal samples from CD and from controls with SEB, a pan T cell stimulator, I observed a significant increase of FOXP3$^+$ cells in both of them. This observation indicates that despite the obeservation that gliadin is able to induce an increase of the number of FOXP3$^+$ cells in the inflamed mucosa of CD, these cells appear unable to control the inflammatory response and

to maintain intestinal homeostasis. In addition, the results observed when the SAg SEB was used as a stimulus indicate that FOXP3 is not expressed just by Tregs in CD but also by other cells that are proliferating in the mucosa after activation with the superantigen. Given the small size of the sample I could not prove that these $FOXP3^+$ cells are suppressing the pathogenic T cells in the lamina propria of CD mucosa.

Understanding the mechanisms of function of $FOXP3^+$ cells in CD lamina propria may be a useful tool to control the break-down of mucosal tolerance observed in CD. In addition, the identification of more specific Tregs markers should assist to clarify the reason of the increase of $FOXP3^+$ cells in CD mucosa after *in vitro* culture with gliadin

7.3 Modulation of $FOXP3^+$ regulatory T cells after anti TNF-α therapy in Crohn's Disease.

The aetiopathogenesis of Crohn's disease is still uncertain but there is good evidence to indicate that this condition falls into the category of disease associated with defective T cell apoptosis that is a fundamental mechanism of immune homeostasis indispensable to the maintenance of balance in the mucosa (Thompson, 1995).

Without proper control of apoptosis, the complex process regulating proliferation and death during an immune response goes wrong followed by an inappropriate accumulation of pathogenic T cells in the tissues that leads to loss of tolerance against commensal flora antigens and inflammation (Sprent and Tough, 2001).

Key cells also involved in maintaining immunological tolerance, including intestinal homeostasis, are represented by the subset of $CD4^+CD25^+FOXP3^+$ Treg cells that play a crucial role in the maintenance of self-tolerance and in the prevention of autoimmune

disease by regulating the effector T cell response against the self antigens of the commensal flora.

In Crohn's disease the loss of tolerance against commensal flora antigens is translated in an uncontrolled immune response prevalently Th1 with TNF-α as a key cytokine involved in the pathogenesis of the disease. Controlling the TNF-α pathway with Infliximab in Crohn's disease has been translated in clinical benefits. Infliximab as anti-TNF-α blocking agent, works by lessening the Th1 cell-mediated effects of the overactive immune response against commensal flora antigens and seems to restore mucosal tolerance translated in the remission of the disease.

The data presented in this thesis show that in the mucosa of active Crohn's the frequency of $FOXP3^+$ Treg cells was down-regulated compared with controls. After the use of Infliximab *in vivo* in Crohn's patients we observed that the number of $FOXP3^+$ Treg cells was increased. These data give rise to the hypothesis that TNF-α might modulate the number and the activity of Treg cells in Crohn's disease that is involved in the loss of self-tolerance against the commensal flora antigens.

Tregs express TNFR2 (Annunziato et al., 2002), suggesting that TNF may modulate the activity of Tregs directly.

TNF-α as pro-inflammatory cytokine is able to promote activation of Th1 effector cell responses in the lamina propria of Crohn's.

With the data obtained in this thesis I hypothesise another role for TNF-α in the inflammatory response in Crohn's disease, as a down-regulator of $FOXP3^+$ Treg cells bearing TNFR2 receptor.

Interaction of membrane bound TNF-α with TNFR2 sends death signals to Treg cells (Annunziato et al., 2002) and this is what I hypothesized in observing the decrease of Tregs in Crohn's patients mucosa. By blocking their interaction using Infliximab, I observed that the number of FOXP3$^+$Tregs is reversed by the effects of anti-TNF-α on Treg cells.

Thus, with these data I hypothesize that Infliximab by blocking mTNF-α inhibits the killing of FOXP3$^+$ Treg by mTNF-α. This observation, together with the knowledge about the induction of apoptosis of pathogenic T cells, could give rise to an new important role of the immunotherapeutic treatment such as Infliximab in controlling the immunological balance in Crohn's disease.

Even if the exact mechanism of Infliximab mediated killing of mucosal T cells remains to be explored, these results provide insights into the mechanisms that TNF-α operates in Crohn's mucosa as regulator of the function and homeostasis of Treg cells.

Whether induction of apoptosis is the dominant mechanism of action that induces the uncontrolled immune response in Crohn's disease should be ascertained in the near future.

Unravelling of this mechanism may shed light on the control of the pivotal pathogenic mechanisms in Crohn's disease by restoration of the function of Treg cells and in the development of novel intervention strategies.

7.4 Modulation of FOXP3$^+$ cells after anti IL-2R therapy in UC.

The use of steroids as well as targeting the IL-2 signaling pathway with CsA has shown clinical benefits in treatment of UC. However some patients are refractory to these therapies. In a recent clinical trial from our group we observed that in children with fulminating UC poorly responsive to combined therapy with steroid and CsA, a good

clinical and histological improvement was observed when they were treated *in vivo* with an immunosuppressant such as anti-IL2R antibody Basiliximab. Treatment with Basiliximab *in vivo* in the patients used in this study led to complete clinical remission in 10-14 days. Blocking the IL-2R with Basiliximab, the pro-inflammatory cytokine IL-2 is not able to bind its receptor and hence T cell proliferation is inhibited. IL-2R is also present on Treg cells so the interruption of the IL-2/IL-2R signaling pathway was able to block not only the pathogenic T cells but also the Tregs.

The result of my study in fact showed that the number of $FOXP3^+$ cells was significantly decreased. As a result of a decrease of $FOXP3^+$ Tregs one could have expected a worsening of the disease instead of an improvement. This result leads me to hypothesize that the number of pathogenic T cells in UC mucosa of these patients is so high that blocking their proliferation is translated into clinical benefits. Because this study was a trial, the number of specimens was very low and I was unable to analyze the ratio between the initial number of effector pathogenic T cells and $FOXP3^+$ Tregs to conclusively prove my hypothesis. Also given the low number of samples I was unable to study the functionality of the $FOXP3^+$ Tregs. An alternative explanation to the observed results could in fact be that $FOXP3^+$ Tregs are not functionally regulatory in UC mucosa. Further studies are needed to shed light on the complexities of mucosal homeostasis that are taking place in UC mucosa and in the understanding of the mechanisms that are needed to control it.

Although FOXP3 appears to be required for human TReg cell development and function, FOXP3 expression is not synonomous with regulatory function, as a significant percentage of human activated T cells express FOXP3 but do not possess regulatory activity (Allan et

al., 2007; Wang et al., 2007). The conclusions of this thesis should be tempered by an appreciation of the technical limitations of investigations in humans.

In this study for example at the beginning I had a look at the Tregs population as a percentage of CD4+CD25+FOXP3+ cells by isolating these cells from the intestinal lamina propria and analyse the data by flow cytometry.

The data obtained showed that FOXP3 was expressed in all the CD4+CD25+ population isolated. Unfortunately, due to the small size of the samples the number of T reg cells obtained was not sufficient to get enough conclusions and from that the decision to use FOXP3 as sole marker to identify Tregs in the mucosa.

The use of alternative markers such as the IL-7 receptor (CD127) (Liu et al., 2006) for example could have been useful to distinguish between the effector and the Treg population. CD127 in fact is down-regulated on the subset of human peripheral FOXP3+ cells. This could have given an answer to the many questions addressed in this thesis regarding the use of FOXP3 as useful tool to analyse the population of Treg cells present in the intestinal mucosa of CD and IBD the patients.

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